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FORMATION AND SIGNIFICANCE OF N-CHLORO COMPOUNDS IN WATER SUPPLIES

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16. ABSTRACT

Many naturally occurring nitrogenous organic compounds readily react with aqueous chlorine, exerting significant chlorine demands. Several N-organic compounds also produce chloroform upon reaction with chlorine with maximum formation occurring between pH 8.5 and pH 10.5. The correlation between chloroform formation and chlorine demand, however, is tenuous. It also appears that intermediates may be formed under neutral or slightly acidic conditions which produce chloroform upon exposure to more alkaline conditions.

Available analytical methods used to differentiate between free and combined chlorine are subject to interference from organic chloramines. Some differentiation, however, may be achieved using amperometric titration.

Seven N-organic compounds were identified in municipal water supplies (adenine, 5-chlorouracil, cytosine, guanine, purine, thymine, and uracil) at concentrations ranging from 20μg/L to 860μg/L. A large unidentified group of primary amine compounds was observed in all of the samples. Field and laboratory data suggested that summer algal bloom occurrences add considerably to the organic nitrogen content of a water supply.

Calculated levels of $CHCl_3$ which might have formed in the water supplies under alkaline conditions were more than 10% of EPA's maximum contaminant level for trihalomethanes. Calculated levels of combined forms of chlorine yielding falsely positive tests for free chlorine in some samples were significant.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This report addresses the hygienic and aesthetic importance of nitrogenous organic compounds in natural waters and the formation and properties of their chlorinated derivatives. The areas studied include: (1) an investigation of the reactivity toward aqueous chlorine of selected nitrogenous compounds analogous to those anticipated as likely constitutents of raw drinking water supplies; (2) an attempt to isolate, concentrate and identify nitrogenous compounds actually present in such supplies as an aid to understanding reactions occurring with nitrogen-containing materials during water chlorination; (3) an evaluation of several analytical methods now in use for distinguishing between free available chlorine and combined chlorine; and (4) the determination of nitrogenous precursors to haloform formation.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

ABSTRACT

A secure hygienic quality in water supplies is dependent upon the maintenance of a free chlorine residual. The available analytical methods used to differentiate between free and combined chlorine, however, are subject to interference from organic chloramines, though it appears some differentiation is achieved using the amperometric method of analysis.

A large number of naturally occurring nitrogenous organic compounds readily react with aqueous chlorine, exerting significant chlorine demands. Several of these compounds also produce chloroform upon reaction with chlorine with maximum formation occurring between pH 8.5 and pH 10.5. The correlation between chloroform formation and chlorine demand, however, is tenuous. It also appears that intermediates may be formed under neutral or slightly acidic conditions which produce chloroform upon exposure to more alkaline conditions.

An analytical scheme was developed to identify trace quantities of N-organic contaminants in dilute aqueous solution. This involved: (1) selective removal of non-nitrogenous compounds using XAD-8 and Tenax GC macroreticular resins; (2) concentration of water samples from 1,000 to 2,000 fold by flash evaporation and lyophilization; and (3) separation and identification by high performance liquid chromatography (HPLC). Seven N-organic compounds were identified in municipal water supplies (adenine, 5-chlorouracil, cytosine, guanine, purine, thymine, and uracil) at concentrations ranging from 20μg/L to 860μg/L. All of the samples exhibited a large unresolved group of compounds rapidly eluting from the chromatographic column. Exhibition of a corresponding fluorescamine responsive fluorescent peak, determination of the organic nitrogen content of this group of materials, and retention positions of reference compounds suggested that this group was composed of primary amine compounds. The demonstration of a parallel increase in organic nitrogen content with population density in two laboratory grown blue-green algal cultures, and the finding of elevated organic nitrogen values in a water supply sample collected during the occurrence of a blue-green algal bloom, suggested that summer algal bloom occurrences can add considerably to the organic nitrogen content of a water supply.

The levels of chloroform which might be formed at pH 7 were calculated by assuming the N-organic compounds identified in the water supplies were not removed prior to chlorination and yielded $CHCl_3$ according to the results of Baum (10). The calculated $CHCl_3$ levels were well below the maximum contaminant level (MCL) of 0.1 mg/L proposed by EPA for total trihalogenated methanes. The calculated $CHCl_3$ formed under more alkaline conditions, however, was more than 10% of the MCL and was therefore significant. The calculated levels of combined forms of chlorine yielding falsely positive tests for free chlorine in

some samples were slightly less or exceeded the 0.5 mg/L free chlorine residual generally taken as an acceptable level of disinfection. If a major portion of the free chlorine residuals determined in finished drinking water consists of the less germicidal combined forms, additional chlorine should be added to assure protection of public health.

This investigation of the formation and significance of organic N-chloro compounds in chlorination of water supplies funded under research grant R803631 involves the identification and determination of nitrogenous organic materials in natural waters and the elucidation of their behavior with aqueous chlorine. The areas studied include: (1) an investigation of the reactivity toward aqueous chlorine of selected nitrogenous compounds analogous to those anticipated as likely constituents of raw drinking water supplies; (2) an attempt to isolate, concentrate and identify nitrogenous compounds actually present in such supplies as an aid to understanding reactions occurring with nitrogen-containing materials during water chlorination; (3) an evaluation of several analytical methods now in use for distinguishing between free available chlorine and combined chlorine; and (4) the determination of nitrogenous precursors to haloform formation.

This report was submitted in fulfillment of Grant # R-803631 by Harvard University under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period April 1, 1975 to June 30, 1979, and work was completed on June 30, 1979.

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SECTION 1

INTRODUCTION

Chlorine was first used in America to disinfect a municipal water supply in 1908 in Jersey City, New Jersey (1). Since then it has become the primary defense against the transmission and spread of waterborne disease. It has been known for the past 60 years or more that free aqueous chlorine ($HOCl$ and OCl^-) reacts readily with ammonia or other nitrogenous organics to form combined forms of chlorine generically termed chloramines. In general, these compounds are much less germicidal than free aqueous chlorine. A secure hygienic quality in water supplies is therefore, dependent upon the maintenance of a free chlorine residual. The combined forms, however, retain an oxidizing capacity and tend to react similarly with many analytical reagents for active chlorine. When these N-chloro compounds are formed, tests for free chlorine may be falsely positive and may indicate a nonexistent germicidal or virucidal behavior.

The ability of varying analytical methods to differentiate between free and combined chlorine is therefore of great importance and was investigated in this study.

It has not always been recognized that there are two types of problems concerned in chemical selectivity of the sort that is required to differentiate between free and combined chlorine. The first type, the one commonly recognized, is that the reagent must not react directly with the combined form of chlorine as it does with the residual free chlorine. But, it must also be kept in mind that even though the reagent reacts only with free residual chlorine, it may nonetheless give a false positive response when hydrolysis of the type reaction (1) occurs.

$$RNHCl + H_2O \rightarrow RNH_2 + HOCl \quad . \qquad (1)$$

Reaction (1) is rapid compared with the time required for the chemical determination.

Differentiation of combined and free residual chlorine is relatively easy when the only combined forms of active chlorine are the ammonia chloramines, for then the hydrolysis equilibria lie sufficiently to the left to make selectivity secure on a thermodynamic basis. The situation is more complex, however, when organic chloramines are concerned, for then hydrolysis may be rapid enough and great enough for response as if there were much free residual chlorine present. But only the instantaneously present free residual chlorine is effective virucidally, not that generated by hydrolysis in response to chemical reaction.

A recent investigation of particular significance in this regard is the study by Guter, Cooper and Sorber (2). Their evaluation of field methods for determining free active chlorine, which included variations on most of the standard techniques, showed that only one, a technique based on the use of syringaldazine, was free of false positive tests as compared with amperometric investigations. So, most methods for determining free active chlorine may give misleading information about the bactericidal or virucidal effectiveness associated with specified chlorination procedures.

An even more intriguing aspect of their work, however, was the finding that the false positives occurred only with organically polluted water and not for synthetic waters containing ammonia. The implication is that organic N-chloro compounds are formed when polluted waters are chlorinated and that some of these react more readily with supposedly selective reagents for free active chlorine than do the ammonia-chloramines.

Even this study had its ambiguities, however, for it was necessary to assume that the amperometric method gives reliable results for free residual chlorine, an assumption that may not always be correct. Only purely physical methods that can measure the free active chlorine instantaneously present in the aqueous solution without disturbance of the labile equilibria can provide thoroughly reliable standards against which proposed chemical determinations can be checked.

The problem has been solved, in part, by the recent work of O'Brien (3), in which all the hydrolysis and ionization constants were evaluated for the chlorinated isocyanurate system. As a result, it is now possible to compute, on purely thermodynamic grounds, the exact concentration of free active chlorine instantaneously present in any chlorinated isocyanurate solution. Indeed, it is possible by adjustment of chlorine to isocyanurate ratio and pH to prepare solutions with desired known concentrations of free and combined residual chlorine. Unfortunately, conclusions reached on the basis of this organic-chloramine system may not be valid for all organic chloramines.

There have been numerous investigations of the reactions of free aqueous chlorine with ammonia, epitomized by the papers of Palin (4) and of Wei and Morris (5). Studies like these have provided reasonably accurate information about the conditions of formation and properties of the ammonia-chloramines that their behavior and germicidal effectiveness relative to free aqueous chlorine can be considered satisfactorily known. Moreover, it has been the custom to check the performance of new analytical methods supposedly specific for free chlorine against prepared solutions of ammonia-chloramines in the assessment of interferences.

In contrast, knowledge about the organic nitrogenous compounds likely to be found in raw waters or about the reactions of these compounds with aqueous chlorine and the properties of the products is almost nonexistent. This is despite the fact that the concentration of organic amine-type nitrogen is likely to be considerably greater than that of free ammonia in natural surface waters not recently contaminated with municipal sewage. A review of older books on water chemistry published in the times when nitrogen analyses were still used as prime indicators of water quality, such as Thresh and

Suckling, *Examination of Waters and Water Supplies* (6), or Mason and Buswell's *Chemistry of Water Supply and Treatment* (7), shows that expected concentrations of free ammonia in upland surface waters are less than 0.06 mg/l N, whereas total organic amino-N measured by the Kjeldahl methods may be expected to be several tenths of a milligram per liter.

In addition to the disinfecting problems caused by the less germicidal N-chloro compounds discussed previously, nitrogenous organics have been shown to produce complex stable mutagenic chlorinated products when reacted with chlorine (8,9). They have also been suggested as possible precursors in haloform formation (10).

Although the composition and extent of hydrocarbon contamination in natural waters has been under extensive examination, the identification of nitrogenous organics in water supplies has not been significantly pursued. This is because many nitrogenous organics are relatively non-volatile and consequently their identification and analysis by gas chromatography is not possible without the prior formation of volatile derivatives. Amino acids are known to be present in the nitrogenous organic fraction found in natural waters, and some attempts have been made to determine them (11-13), but the quantities found--some µg per l of N *in toto*--seem small compared with total organic-N expected. One would also expect considerable concentrations of pyrrolic-N from the breakdown of chlorophyll and similar plant compounds, of purine derivatives and of pyrimidine-related compounds such as uracil and other nucleic acid components, but there appears to be little specific information available.

Recent advances in the field of high pressure liquid chromatography (HPCL), have made the detection of non-volatile nitrogenous compounds increasingly feasible. Amino acids commonly found in protein hydrolysates, physiological fluids (14) and standard amino acid mixtures (15-25) have been resolved using HPLC with fluorescence detection at the picomole level. Dr. R. Jolley and Dr. W. Pitt have made significant progress in the separation and identification of trace organic compounds in urine (26), primary and secondary stages of municipal sewage treatment plants, and natural waters (27-29) using a strongly basic anion exchange resin (Bio-Rad aminex A-27) with ammonium acetate acid buffer eluent. Jolley (30,31) also found that a number of chlorinated pyrimidines and purines were formed during the chlorination of sewage effluent. Among these were 5-chlorouracil, 5-chlorouridine, 8-chlorocaffeine, 6-chloroguanine and 8-chloroxanthine.

There have been some studies on the reactions of amino acids with aqueous chlorine, but very little on reactions of other probable aqueous nitrogenous compounds. The survey by Tarras (32), although widely quoted, provides little positive information. Palin (4) extended his work with ammonia to include some observations of breakpoint phenomena. Culver (33), made a detailed study of reactions in the chlorination of glycine, finding, among other things, that cyanogen chloride could be formed as an intermediate product. Friend (34), showed that many amino-acids gave N-chloro derivatives rapidly, more rapidly than NH_3 at neutral pH. Conditions of formation and properties of many other N-chloro compounds are known, of course, but not of those that are likely to be of interest in connection with water chlorination.

One category of reactions between chlorine and aqueous nitrogenous compounds of particular concern is that which produces haloforms. The discoveries by Rook (35) and by Bellar *et al.* (36) that the chlorination of water supplies containing organic matter produces chloroform and other trihalomethanes, have evoked concern because of the potential carcinogenic and toxic properties of these compounds. Morris (37) calculated the intake of a 50 kg individual consuming one liter per day of water containing 0.1 mg/l of chloroform to be .002 mg/kg, about one two-hundredth of the minimum observed chronic toxicity level. It was suggested (38) that compounds which ionize rapidly to give carbanions account for the formation of chloroform within the contact time of most water treatment plants and distribution systems. One structure in which active carbanion formation occurs is the nitrogen containing pyrrole ring. The hydrogens ortho to the nitrogen are activated like those in phenol and provide sites for chlorination and subsequent haloform formation. The pyrrole ring is naturally important because of its occurrence in chlorophylls, xanthophylls and heme. Indole and derivatives like tryptophan also contain pyrrolic units. An investigation into the haloform producing potential of selected nitrogenous organics was therefore pursued in this study.

This report addresses the hygienic and aesthetic importance of nitrogenous organic compounds in natural waters and the formation and properties of their chlorinated derivatives. Such information is important hygienically because assured disinfection of all forms of pathogens can be maintained reliably only if specific determinations for free aqueous chlorine are not subject to interference by organic N-chloro compounds that may be formed. It is also important aesthetically because it appears that some of the noxious tastes and odors commonly attributed to aqueous chlorine may actually be due either to organic N-chloro compounds or to NCl_3 formed from such compounds under conditions where it is not normally formed from NH_3.

This investigation of the formation and significance of organic N-chloro compounds in chlorination of water supplies funded under research grant R8036301 involves the identification and determination of nitrogenous organic materials in natural waters and the elucidation of their behavior with aqueous chlorine. The areas studied include: (1) an investigation of the reactivity toward aqueous chlorine of selected nitrogenous compounds analogous to those anticipated as likely constituents of raw drinking water supplies; (2) an attempt to isolate, concentrate, and identify nitrogenous compounds actually present in such supplies as an aid to understanding reactions occurring with nitrogen-containing materials during water chlorination; (3) an evaluation of several analytical methods now in use for distinguishing between free available chlorine and combined chlorine; and (4) the determination of nitrogenous precursors to haloform formation.

A detailed literature review on the occurrence and environmental significance of nitrogenous organic contaminants in water, and analytical methods for their determination is included in Appendix C.

SECTION 2

SUMMARY

1. All of the methods used to measure free chlorine (DPD, orthotolidine-arsenite (OTA), LCV, SNORT, SYRING, and amperometric titration) are subject to interference from organic chloramines.

2. The amperometric method displays some differentiation between free and total chlorine.

3. If only amino acids are present along with ammonia, the DPD or SYRING methods should be used since these appear to be the most specific for free chlorine under these conditions.

4. XAD and Tenax resins selectively remove non-nitrogenous organic contaminants from water although not with 100% efficiency.

5. A large number of naturally occurring nitrogenous organic compounds readily react with aqueous chlorine, exerting significant chlorine demands.

6. Several nitrogenous organic compounds produce chloroform upon reaction with chlorine.

7. Chlorine demand does not appear to be a very good indication of the chloroform producing potential of an individual compound.

8. For chloroform products, increasing the pH has a considerable influence on the quantity of chloroform formed. Maximum chloroform formation occurs between pH 8.5 and pH 10.5.

9. Compounds that show significant chloroform production only under alkaline conditions may form other chlorinated derivatives under neutral or slightly acidic conditions. These intermediates produce chloroform upon exposure to more alkaline conditions.

10. Chlorination may have the beneficial effect on chemical water quality in reacting to eliminate amines which are natural precursors of carcinogenic nitrosamines formed by reaction with salivary or gastric nitrite.

11. Chromatograms of resin-filtered samples showed no improvement in either U.V. or fluorescence background traces over chromatograms of unfiltered samples. The total number of compounds identified at a given collection site was maximized, however, by analyzing both raw and resin-filtered samples. This relates to item #4.

12. The average values for compounds identified in the water supply samples were 367, 60, 20, 167, 200, 110, and 160 μg/L, for adenine, 5-chlorouracil, cytosine, guanine, purine, thymine and uracil, respectively. Uracil, adenine, and guanine were found most frequently in the water supply samples while 5-chlorouracil, cytosine and purine were only encountered once in these sources.

13. Summer algal blooms may contribute significantly to the organic nitrogen content of water supplies.

14. The levels of chloroform which might be formed at pH 7 were calculated by assuming N-organic compounds identified in the water supplies were not removed prior to chlorination and yielded $CHCl_3$ according to the results of Baum (10). The calculated $CHCl_3$ levels were well below the maximum contaminant level (MCL) of 0.1 mg/L proposed by EPA for total trihalogenated methanes. The calculated levels of $CHCl_3$ formed under more alkaline conditions, however, were more than 10% of the MCL and were therefore significant.

15. The calculated levels of combined forms of chlorine yielding falsely positive tests for free aqueous chlorine in some samples were slightly less or exceeded the 0.5 mg/L free chlorine residual generally taken as an acceptable level of disinfection. If a major portion of the free chlorine residuals determined in finished drinking water consists of the less germicidal combined forms, additional chlorine should be added to assure protection of public health.

SECTION 3

DIFFERENTIATION BETWEEN FREE AND COMBINED CHLORINE

As discussed previously combined available chlorine is a much less active disinfectant than free chlorine. Analytical methods capable of differentiating between free available chlorine and the various forms of combined chlorine and the various forms of combined chlorine are, therefore, needed to ensure that water supplies are adequately disinfected. However, interconversion of these species occurs in solution through the hydrolysis of combined chlorine to free chlorine (equation (2)), thus presenting difficulties in developing suitable analytical methods.

$$R_2NCl + H_2O \rightarrow R_2NH + HOCl \quad . \qquad (2)$$

The methods currently most used for differentiating free chlorine from the combined forms are DPD (39-43), orthotolidine-arsenite (OTA) (40,44,45), LCV (39,46,47), SNORT (39,48), SYRING (39,48,51), and amperometric titration (39,40,52-58). These methods rely upon the more rapid rate of reaction between the given reagent with HOCl as compared to the N-chloro compound (kinetic selectivity), and on the slowness of hydrolysis of the N-chloro compound.

The errors involved in the kinetic selectivity of the analytical reagents are illustrated by their reaction with NH_2Cl and $NHCl_2$. DPD and SNORT, for example, react with these chloramines at a rate of 3.6% and 1.1% per minute, respectively. The reaction with SYRING is slower, however, with interference only being observed at higher NH_2Cl concentrations (48,49). The selective differentiating ability of the analytical reagents decreases (40,59,60) at lower pH values. This may be partially attributable to the acid catalysis of the hydrolysis reaction (equation (2)) (61,62) as illustrated by the inability of the acid orthotolidine method to distinguish between free and combined chlorine without rapid addition of arsenite.

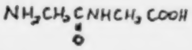
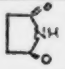
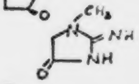
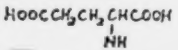
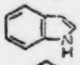
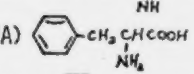
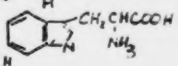
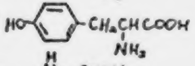
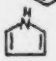
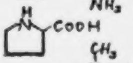
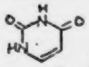
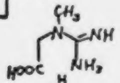
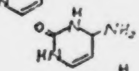
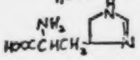
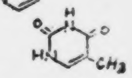
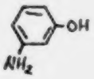
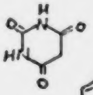
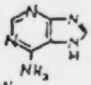
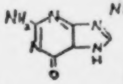
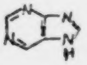
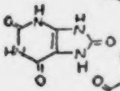
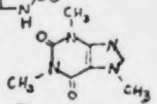
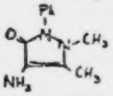
Several of the analytical reagents have been tested for their response to N-chloro compounds. Palin (4) found that DPD could distinguish between free chlorine and several types of chlorinated amino acids. The OTA procedure was found to distinguish between free and combined oxidant when seawater containing several amino acids, diphenylamine and uracil was chlorinated (65).

In studying the SYRING procedure, it was found that although it worked well in simple solutions of known composition, there were several reports of false positive chlorine in natural waters (49,50). It was also found that N, N^1-2, 2^1-4, 4^1-6, 6^1-octachloro-N, N^1-diphenylurea reacted with SYRING (66).

Recently, a membrane electrode has been developed by Johnson (67,68) which shows promise as a discriminatory method. Selectivity is based on species volatility and the application of an appropriate positive potential to the electrode.

The experimental work on this topic examined the selective differentiating ability of the six analytical reagents for free aqueous chlorine in the presence of a variety of N-chloro compounds. Several types of nitrogenous organic compounds were investigated and are shown in Table 1.

TABLE 1. NITROGENOUS ORGANIC COMPOUNDS STUDIED

AMINO ACIDS			HETEROCYCLIC BASES		
GLYCYLGLYCINE	(GLY)	 $NH_2CH_2\underset{O}{C}NHCH_2COOH$	SUCCINIMIDE	(SU)	
SARCOSINE	(SA)	CH_3NHCH_2COOH	CREATININE	(CRE)	
GLUTAMIC ACID	(GLU)	 $HOOCCH_2CH_2\underset{NH}{CH}COOH$	INDOLE	(IN)	
PHENYLALANINE	(PA)		TRYPTOPHAN	(TRY)	
TYROSINE	(TY)		PYRROLE	(PY)	
PROLINE	(PR)		URACIL	(U)	
CREATINE	(CR)		CYTOSINE	(C)	
HISTIDINE	(H)		THYMINE	(T)	
M-AMINOPHENOL	(AP)		BARBITURIC ACID	(B)	
			ADENINE	(A)	
			GUANINE	(G)	
			PURINE	(P)	
			URIC ACID	(UR)	
			CAFFEINE	(CA)	
			4-AMINO-ANTIPYRINE	(AAP)	

EXPERIMENTAL METHODS

Chlorine-Demand Free Water (CDFW)

All solutions used in this study were prepared using chlorine-demand free water, prepared by dosing distilled water with 1-2 mg/l chlorine and and destroying the chlorine remaining after 6-8 hours by UV radiation. All glassware was dosed with 1-2 mg/l chlorine for several hours and rinsed with CDFW.

Chlorine Solutions

Concentrated chlorine solution was prepared by bubbling chlorine gas into distilled water, adjusting the pH to 10 with KOH and then distilling this solution at 90°C under suction. This produced a 10^{-2} M solution of chloride-free HOCl. A 1×10^{-3} M stock solution of HOCl was prepared by dilution and stored in a low actinic glass bottle at 5°C. Total available chlorine in this solution was determined periodically using the thiosulphate-iodide titration method.

Buffer Solutions

A 10^{-3} M sodium bicarbonate buffer solution in CDFW was used in which the pH was adjusted to pH 7.0 using CO_2 so that the chloride concentration could be kept as low as possible.

10^{-3} M Stock Solutions of Nitrogenous Compounds

The samples of compounds investigated were of the highest quality commercially available, and were not purified further before use. The compounds were dissolved in 10^{-3} M pH 7.0 bicarbonate buffer.

Analytical Methods

The methods employed were DPD, ORTHO (and later OTA), LCV, SNORT, SYRING and amperometric titration. The reagents were prepared according to Standard Methods (39). Color development was measured on a Beckmann DU Spectrophotometer. A Fisher Model 393 Chlorine Titrimeter was used for amperometric titrations. Reagents were discarded if they became discolored or were found to have lost sensitivity to the stock chlorine solution. The limit of detection of the colorimetric methods varied between batches of reagents from about 0.02 mg/l to 0.15 mg/l; the limit of detection for the amperometric method was about 0.05 mg/l.

Analytical Procedures

10 ml of a 10^{-3} molar concentration of each compound tested was dosed with sufficient 1×10^{-3} M stock chlorine solution to give an initial molar chlorine to compound ratio of 1:1, 2:1 or 3:1. Reaction was allowed to proceed for half an hour in the dark, at which time the mixture was diluted to 1000 ml with 10^{-3} M pH 7 buffer and transferred to an amber bottle where it was mixed thoroughly for another 15 minutes. A portion of the solution was then added to the analytical reagent and analysed as rapidly as possible.

Analysis using DPD, ORTHO, LCV, SNORT, SYRING and amperometric titration was carried out, in that order, a process which generally took 1 to 1-1/2 hours. Very little change in residual chlorine was found to occur during this time. In the later experiments with cyanuric acid, the reaction procedure was altered so that the reactions could be carried out at 10^{-5} M concentrations instead of 10^{-3} M concentrations. 500 ml solutions containing the appropriate amount of the compound and chlorine were mixed in less than 30 seconds from individual separatory funnels leading directly into an amber bottle, where the solution was thoroughly mixed for 45 minutes before analysis.

CHLORINATED CYANURIC ACID

Considerable emphasis in the investigation of the analytical methods was focused on the cyanuric acid/aqueous chlorine system because of the ability to predict the equilibrium composition of the solution from the equilibrium constants measured by O'Brien *et al.* (69), or by Pinsky and Hu (70). At equilibrium in aqueous solution, mono, di and trichloro derivatives coexist along with substantial quantities of free chlorine (F).

Aqueous chlorine was mixed with cyanuric acid and solutions of the chlorinated cyanuric acids were also prepared. The results of the analysis of free (F) and total (T) chlorine under different conditions at each stated chlorine: cyanuric acid molar ratio are presented in Tables 2 to 4. This is compared with the theoretical concentrations of free chlorine (F), the monochlorinated species (M), the dichlorinated species (D) and total chlorine (T) at pH 7.0, derived from O'Brien (3).

There is considerable scatter in the concentration measured by each analytical method with a standard deviation of about 10-15%. There is also some variation between the categories. This may be due to (i) differences in mixing and thus demand, or (ii) low reproducibility in the analytical method.

The results indicate that, with the exception of the amperometric method:

(i) none of the methods for free chlorine can distinguish between free and combined chlorine;

(ii) all methods measure the total available chlorine.

The amperometric method displayed some differentiation between free and total chlorine, indicating that with at least some of the combined species, hydrolysis is slow. The interference observed in the other methods for free chlorine is, therefore, caused by the reaction of the analytical reagent with the N-chloro compound. In the amperometric method, this reaction appears to be slow, while in the colorimetric methods it is rapid.

TABLE 2. CHLORINATED CYANURIC ACID

MOLAR RATIO 1:1 0.71 MG/L CL_2 pH 7.0
RESIDUAL AVAILABLE CHLORINE (MG/L)[a]

Reaction Condition	Species Measured	METHOD					
		DPD	ORTHO	LCV	SNORT	SYRING	AMP
10^{-3}M Reaction	F	0.55	0.55	0.45	0.50	0.55	0.40
	T	0.50	0.60	0.55	0.55		0.50
10^{-5}M Reaction	F	0.40	0.40	0.60	0.50	0.45	0.40
	T	0.50	0.40	0.45	0.50		0.50
Average	F	0.50	0.45	0.55	0.50	0.50	0.40
	T	0.50	0.50	0.50	0.50		0.50
Theory	F			0.33			
	M			0.31			
	D			0.07			
	T			0.71			

[a] Averages in MG/L CL_2, rounded to nearest 0.05 MG/L.

TABLE 3. CHLORINATED CYANURIC ACID

MOLAR RATIO 2:1 1.42 MG/L CL_2 pH 7.0
RESIDUAL AVAILABLE CHLORINE (MG/L)[a]

Reaction Condition	Species Measured	METHOD					
		DPD	ORTHO	LCV	SNORT	SYRING	AMP
10^{-3}M Reaction	F	1.20	1.25	1.45	1.45	1.30	0.40
	T	1.10	1.30	1.20	1.25		0.95
10^{-5}M Reaction	F	1.00	1.00	1.10	1.05	1.00	0.90
	T	1.05	1.00	1.15	1.10		1.05
Dichloro-Cyanurate	F	0.80	0.70	1.00	0.75	0.80	0.80
	T	0.90		0.90	0.80		0.95
Average	F	1.00	1.00	1.25	1.10	1.00	0.75
	T	1.00	1.10	1.10	1.05		0.95
Theory	F			0.79			
	M			0.41			
	D			0.22			
	T			1.42			

[a] Averages in MG/L CL_2, rounded to nearest 0.05 MG/L.

TABLE 4. CHLORINATED CYANURIC ACID

MOLAR RATIO 3:1 2.13 MG/L CL_2 pH 7.0
RESIDUAL AVAILABLE CHLORINE (MG/L)[a]

Reaction Condition	Species Measured	METHOD						
		DPD	ORTHO	LCV	SNORT	SYRING	AMP	ELEC
10^{-3}M Reaction	F	1.85	1.95	2.10	2.05	2.15	1.10	
	T	1.65	2.10	2.05	1.90		1.55	
10^{-5}M Reaction	F	1.60	1.60	1.90	1.75	1.75	1.40	
	T	1.60	1.55	1.80	1.80		1.70	
Trichloro-Cyanurate	F	1.60	1.70	2.15	1.90	1.75	1.60	
	T	1.70		2.05	1.90		1.80	
Average	F	1.65	1.75	2.05	1.95	1.85	1.50	
	T	1.65	1.80	2.00	1.85		1.75	
Theory	F	1.34						
	M	0.41						
	D	0.38						
	T	2.13						

[a] Averages in MG/L CL_2, rounded to nearest 0.05 MG/L.

NITROGENOUS ORGANIC COMPOUNDS

Amino Acids

The results of free chlorine measurement in the presence of amino acids with a 1:1 ratio of aqueous chlorine are presented in Fig. 1. Several patterns are evident in this graph. Zero free chlorine is reported by SYRING in the presence of 6 amino acids (glycylglycine, glutamic acid, sarcosine, phenylalanine, tyrosine and histidine), in contrast to ORTHO, LCV and SNORT. Similarly, DPD reports zero free chlorine in solutions containing 5 of these amino acids (i.e., with the exception of sarcosine). Because DPD, LCV and SNORT report a high total chlorine concentration (see Fig. 2) in these solutions, it seems likely that DPD and SYRING can differentiate effectively between free and combined chlorine in these systems.

At the 2:1 ratio, DPD again appears to be differentiating effectively. However, the trend observed in free chlorine measurements at the 1:1 ratio with the SYRING and SNORT methods are reversed (see Fig. 3). The SYRING method seems to also be measuring combined chlorine especially in those solutions containing glycylglycine, sarcosine and tyrosine.

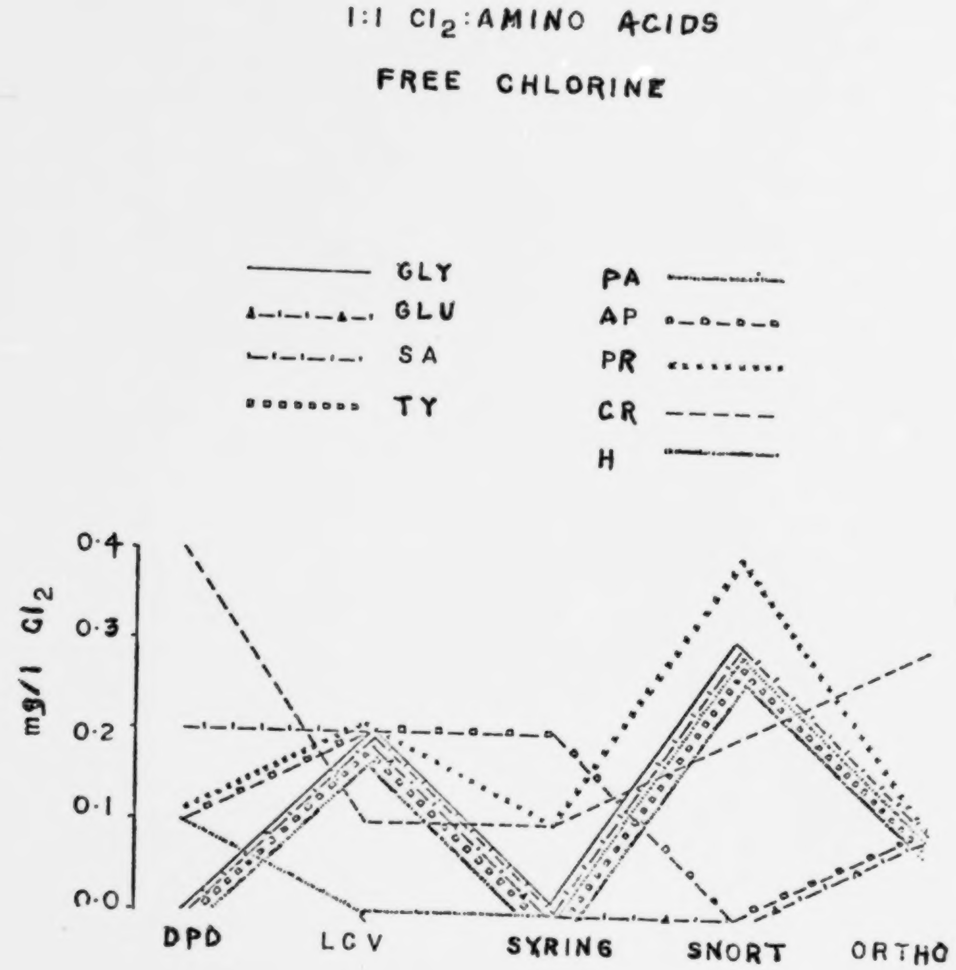


Figure 1. 1:1 Chlorine:amino acids. Free chlorine measured in solution initially containing 0.7 mg/1 Cl_2.

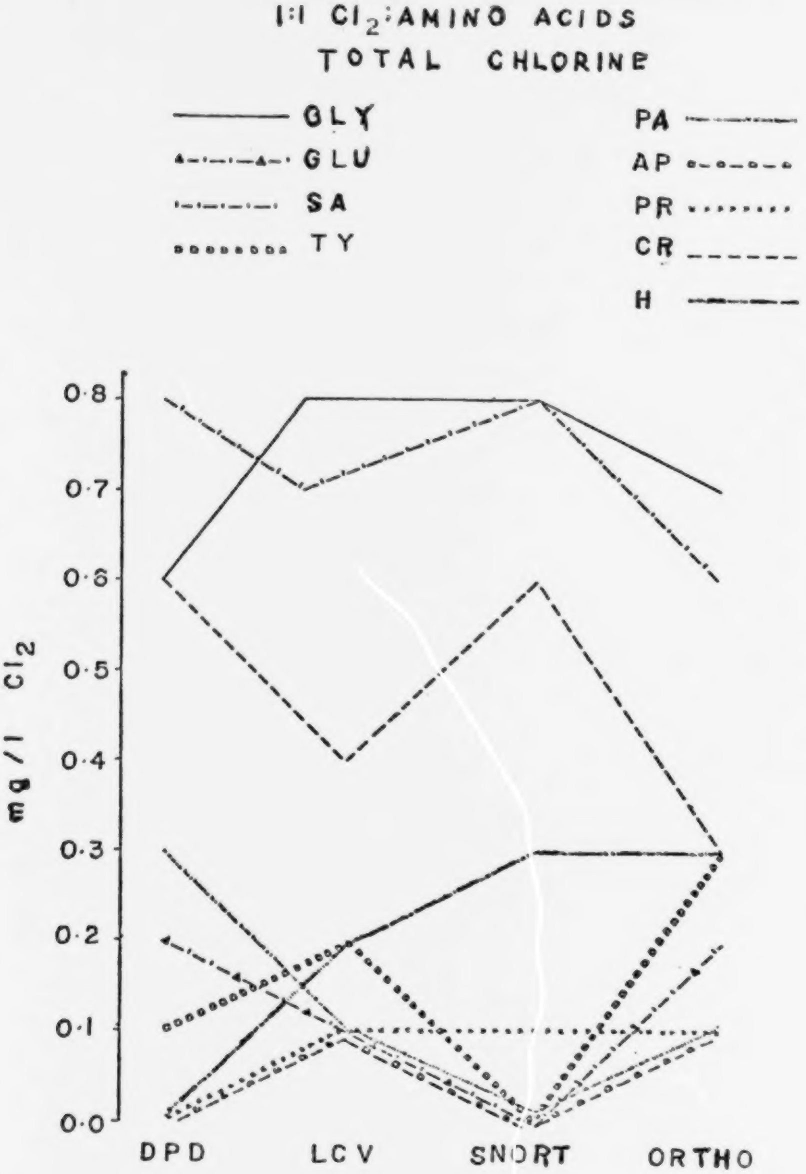


Figure 2. 1:1 Chlorine:amino acids. Total chlorine measured in solution initially containing 0.7 mg/l Cl_2.

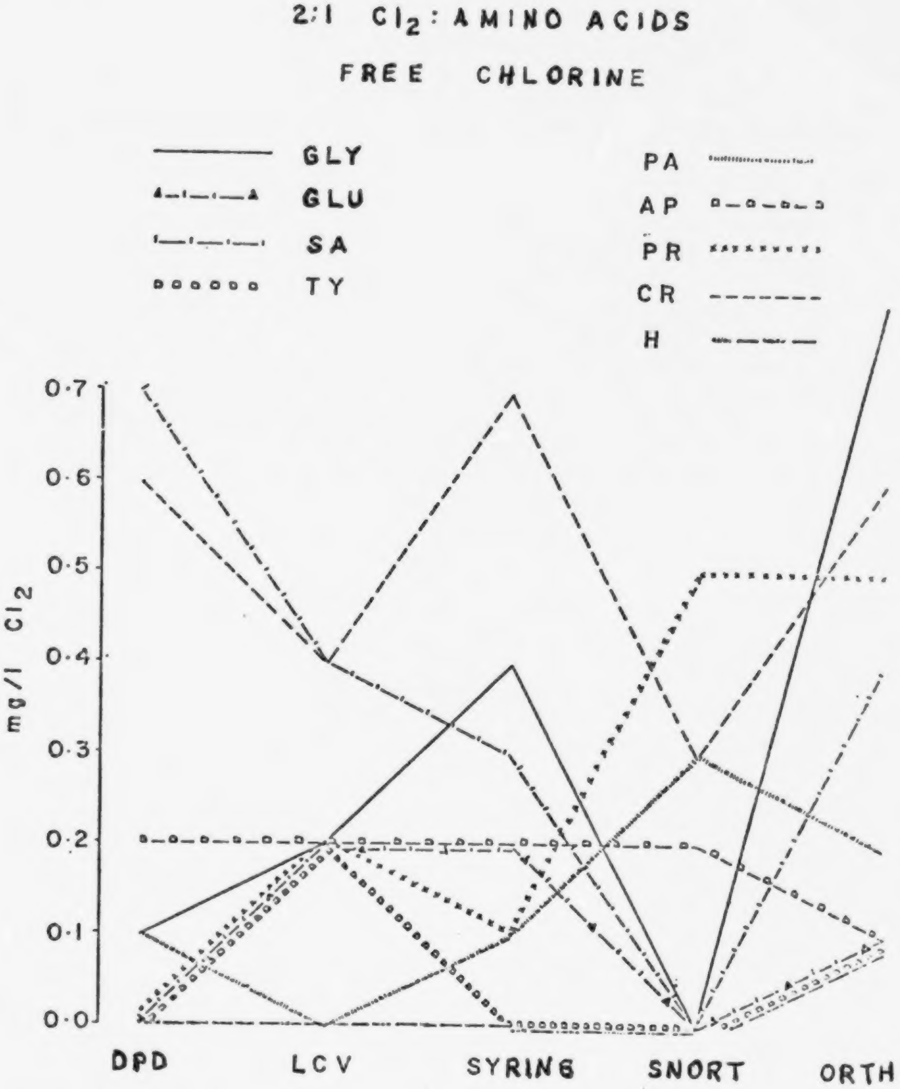


Figure 3. 2:1 Chlorine amino acids. Free chlorine measured in solution initially containing 1.4 mg/l Cl_2.

This study confirmed Palin's finding (4) that DPD was able to differentiate between free chlorine and chlorinated amino acids, although we also found several amino acids (creatine and sarcosine) where such discrimination was not found. Some doubts exist about the capabilities of SYRING and SNORT. It seems SYRING may be able to distinguish between free chlorine and chlorinated amino acids (i.e., with the exception of creatine and sarcosine), while SNORT does not. Moreover, SNORT seemd to suffer from a bleaching of the blue color, formed by free chlorine, when total chlorine was measured (see Fig. 4).

2:1 Cl_2:AMINO ACIDS

TOTAL CHLORINE

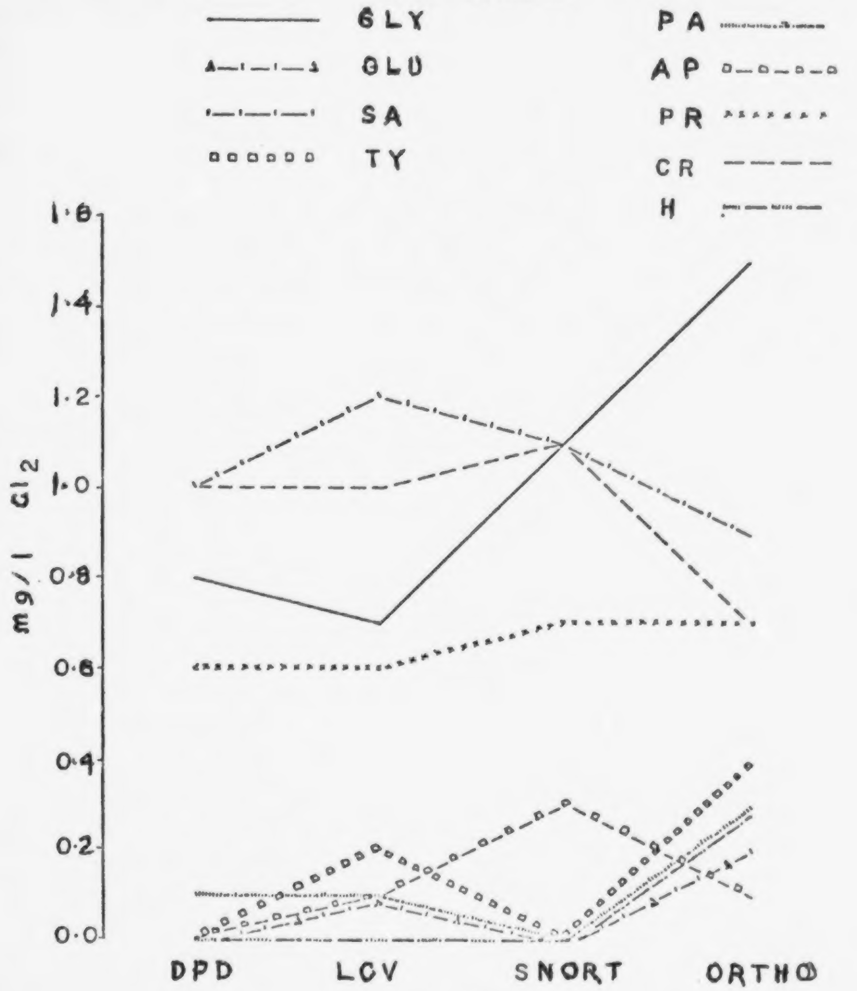


Figure 4. 2:1 Chlorine:amino acids. Total chlorine measured in solution initially containing 1.4 mg/l Cl_2.

ORTHO and LCV measure some combined chlorine in the free chlorine determination. It is therefore surprising that Duursma and Parsi (65) were able to differentiate between free and combined oxidant formed from glycine, alanine, cysteine, leucine and tyrosine using OTA. However, this may indicate the behavior of N-bromo compounds towards the analytical reagents is different from the N-chloro compounds.

Differentiation between free chlorine and chlorinated amino acids thus seems to be a result of the kinetics of hydrolysis. Methods employing a neutral pH can differentiate, but decreasing the pH even to pH 4 increases the rate of hydrolysis sufficiently to cause artificially high determinations of free chlorine.

Heterocyclic Bases

For several of the heterocyclic compounds, chlorine demand was so high that no available chlorine remained in solutions mixed with equal molar ratios of aqueous chlorine. There is not as striking a pattern in the measurement of free chlorine in the presence of these compounds as with the amino acids (see Fig. 6). SNORT does tend to report higher concentrations of free chlorine than the other methods and in most cases, the total and free concentrations reported by SNORT are the same (see Figs. 5 and 6) suggesting it does not differentiate between free and combined chlorine. All the analytical methods are subject to some false positive readings for free chlorine.

Since at least one method measured a zero free chlorine concentration in each solution examined, with the exception of those containing purine or succinimide, hydrolysis of these N-chloro compounds must be slow and interference is due to direct reaction with the analytical reagent.

Selected Amino Acids and Bases

Glycylglycine--
DPD can differentiate between free chlorine and the mono- and di-chloro derivatives, the combined chlorine being measured in the monochloramine fraction in both solutions. SYRING can also differentiate between free chlorine and the monochloro derivative.

Sarcosine--
Since only a mono-chloro derivative is possible, the results shown in Figs. 4 and 5 for the 2:1 ratio indicate this derivative is relatively stable in the presence of excess chlorine. Complete differentiation between this species and free chlorine is only achieved by SYRING.

Creatine--
Lomas (71) carried out some chlorination studies on this compound and found that, using an orthotolidine-ferrous titration procedure (similar to the SNORT procedure at pH 7), he could differentiate between free and combined chlorine. Our results using SNORT show there is some interference of combined chlorine with the free chlorine measurement.

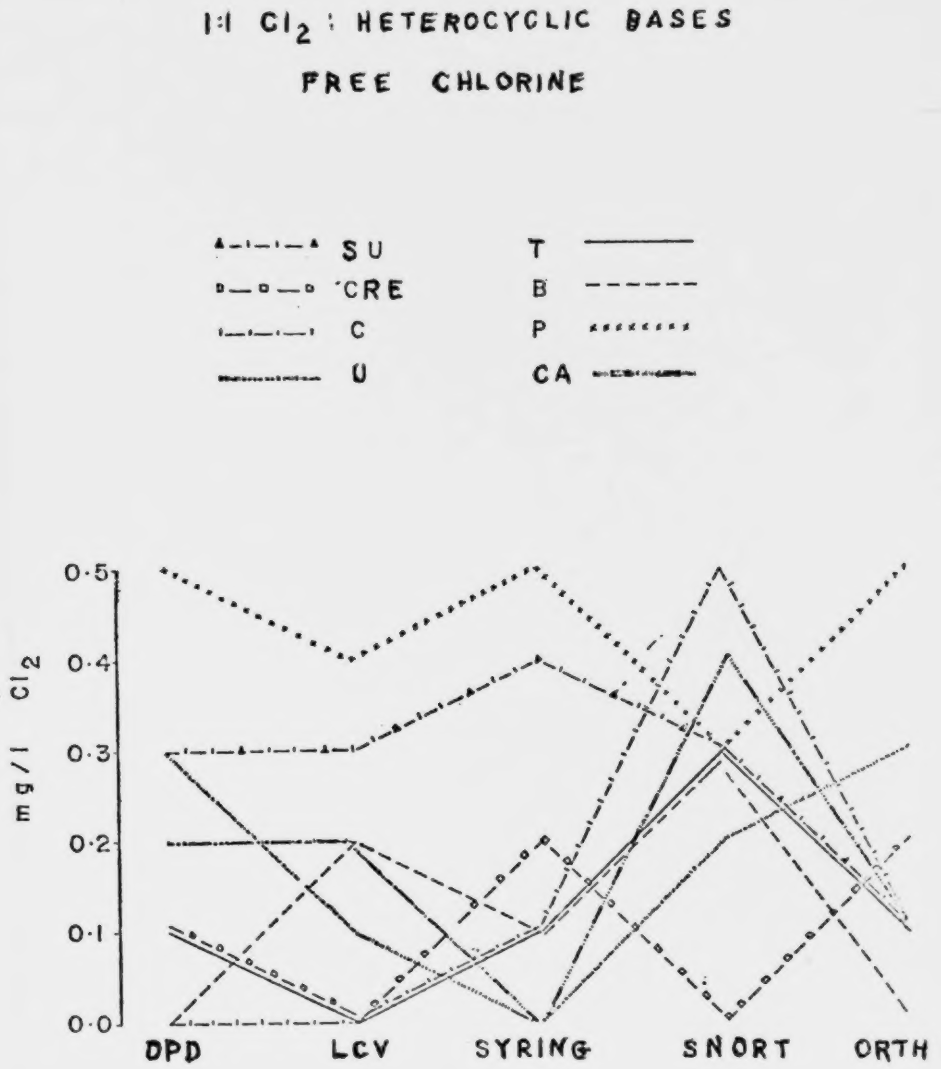


Figure 5. 1:1 Chlorine:heterocyclic bases. Free chlorine measured in solution originally containing 0.7 mg/l.

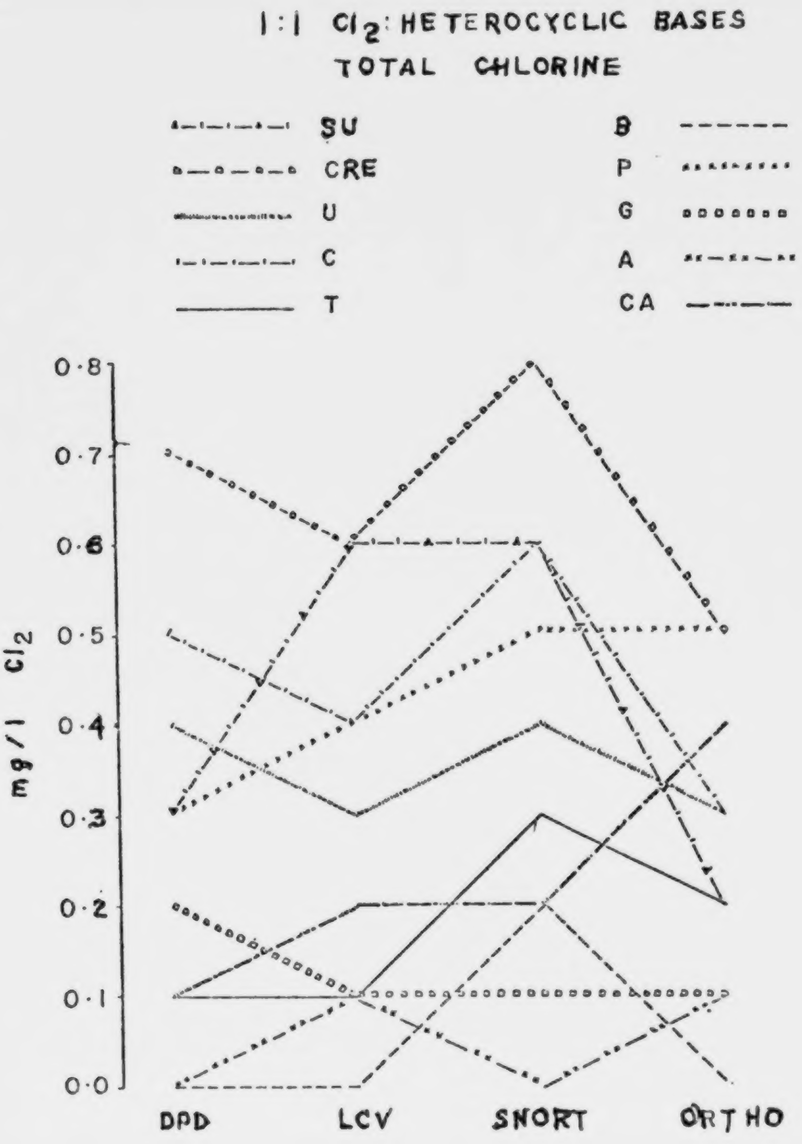


Figure 6. 1:1 Chlorine:heterocyclic bases. Total chlorine measured in solution originally containing 0.7 mg/l Cl_2.

Succinimide--

About 4% of the total available chlorine can exist as free chlorine at equilibrium with N-chloro succinimide. All methods suffer from some interference of N-chloro succinimide. However, ORTHO measures a lower free and total chlorine concentration than the other methods, suggesting the hydrolysis of N-chlorosuccinimide and its reaction with orthotolidine are both slow at pH 1.3.

Creatinine--

SNORT is the only method which is capable of differentiating between free chlorine and the chlorinated creatinines, either the mono- or the dichloro derivative. In the methods were distinction between monochloramine and dichloramine can be made (DPD and SNORT), concentrations of combined chlorine were reported in both fractions. This may be evidence that either derivative reacts readily in the total method, but may slowly interfere in the monochloramine fraction, and even with the measurement of free chlorine. Lomas (71) obtained similar results, both with his "SNORT" procedure and the DPD method. However, he interpreted his results as being evidence for the formation of a mono-chloro derivative only.

Cytosine--

The SNORT method measured combined chlorine species in the dichloramine and monochloramine fractions at high cytosine concentrations using chlorine to cytosine molar ratios of 1 and 2, respectively. This can be explained by the greater reactivity of the dichlorocytosine. At lower cytosine concentrations, however, no differentiation was observed. DPD does differentiate between free and monochlorocytosine, the later being observed as dichloramine. Some dichlorocytosine does interfere in the measurement of free chlorine in the DPD method while the remainder is observed as monochloramine. LCV, however experiences no interference from either of these compounds in the measurement of free chlorine.

CONCLUSIONS

All of the methods currently used to measure free chlorine are subject to interference from organic chloramines. Reliable measurements of the free chlorine present in water supplies or wastewaters containing organic nitrogen can thus not be made unless the types of organic nitrogen compounds present are known. If only amino acids are present along with ammonia, the DPD or SYRING methods should be used since these seem to be the most specific for free chlorine under these conditions.

SECTION 4

IDENTIFICATION OF NITROGENOUS ORGANICS IN WATER SUPPLIES

Until recently the organic content of water was generally evaluated using gross organic analytical determinations such as Total Organic Carbon (TOC), Chemical Oxygen Demand (COD), various extracting methods (CAE and CCE) or Biological Oxygen Demand (BOD). Quantitative determination of individual molecular species present in the microgram per liter range represented a formidable analytical task. The need to understand the specific nature of the array of contaminants present in our water supplies, however, has lead to significant progress in the development of methods and instrumentation required for the identification and quantification of such contaminants. Resins, capable of removing specific categories of trace organics, and the development of high pressure liquid chromatographic techniques have made possible the detection of non-volatile nitrogenous organic compounds from dilute sources.

Quantitative determination of organic constituents involves concentration, separation, and identification techniques. Because of the broad types of organic compounds likely to be present in natural waters the analytical procedure must also include a pretreatment step in which "interfering" non-nitrogenous organic compounds are selectively removed. The remaining organics, present at only microgram per liter concentrations must then be concentrated by factors of a thousand fold prior to chromatographic separation.

The procedure used in this study to identify trace nitrogenous organics involved: (1) the selective removal of interfering, non-nitrogenous organic material using macroreticular resins [(XAD-2,4 and 8) (72), (Tenax) (73), (XE-340, 347, 348) (74)], concentration of water samples by flash evaporative technique followed by freeze drying, and separation and identification of specific compounds using high pressure liquid chromatography.

ANALYTICAL METHODS

Sample Sites and Collection

Field samples were collected from several water supplies of northeastern Massachusetts as well as one from Bethesda, Ohio. The Massachusetts locations were chosen because hydrologic records indicated that summer blue-green algal blooms were likely to occur in these sources and because of their proximity to the research laboratory of this investigator. The municipal water supplies selected for the study were: Billerica, MA (Concord River), Lawrence, MA

(Merrimack River), and Danvers, MA (Merrimack River), and Danvers, MA (Middleton Pond). Other Massachusetts water supplies were considered but the absence of observed algal blooms in the hydrologic records of these sources precluded their inclusion in this study. It was supposed that sampling during a blue-green algal bloom would increase the likelihood of detecting individual N-organic compounds, because organic nitrogen compounds are known to be released as extracellular metabolites by this group of algae. Arrangements were also made for the sampling and shipment of water samples from Fairfax County, VA during bloom occurrences. Plant superintendents at all locations cooperated by reporting the occurrence of any increased algae growth in the water supplies under investigation. Water samples were collected immediately after notification of a bloom occurrence and prior to addition of algicides to the water supply. Samples were also taken from the Marlboro East and West sewage treatment plants to confirm the ability to the analytical procedure to separate and identify organic components from presumably more concentrated sources. Samples from Spy Pond, Arlington, MA, and the Charles River, Cambridge, MA, were also collected.

Non-bloom water samples were collected by grab sampling from approximately the upper six inches of the river or pond using a plastic bucket. The samples were then transferred into several acid-washed (10% HCl) one-gallon polyethylene jugs for transport to the laboratory. The Danvers 'bloom' sample was collected by skimming the upper inch of water from regions of dense algal growth. The sample was collected prior to treatment of the pond with copper sulfate. Water samples taken during the second sampling of the Concord River (Billerica, 7/14/78) were obtained by selectively collecting water from shallow regions containing algal mats or dense aquatic weeds. All river sites with the exception of the second Billerica sampling were located at the intake channels of the water treatment facilities. Pond samples were taken at convenient spots along the shore. Samples collected during the second sampling of the Concord River were taken just downstream of the water treatment plant near Bridge Street and River Street (Figures 7 through 10).

Approximately 15 liters of water were collected at each site. Samples were returned to the laboratory within one hour of sampling where they were either analyzed immediately for ammonia and Kjeldahl nitrogen, or stored at 5 degrees centigrade for subsequent analysis. Filtration and concentrations were carried out within one to five days of the sample collection. Ammonia and Kjeldahl-nitrogen contents were determined no more than 24 hours after collection of the sample.

In addition to the field sites, samples were also taken from laboratory grown cultures of two blue green algae, *Anabaena flos aquae* and *Oscillatoria tenuis* (obtained from the Texas culture collection of algae, Dr. Richard Starr, University of Texas, Austin, TX). These two species were chosen because they are known to liberate large quantities of nitrogenous organic compounds during growth (75,76,77).

The *Anabaena flos aquae* culture was incubated for 42 days while the *Oscillatoria tenuis* culture was grown for 35 days. The cultures were sacrificed, filtered to 0.7 mμ with Whatman GF/F filter paper and concentrated by rotary evaporation followed by lyophilization. Concentrated samples were

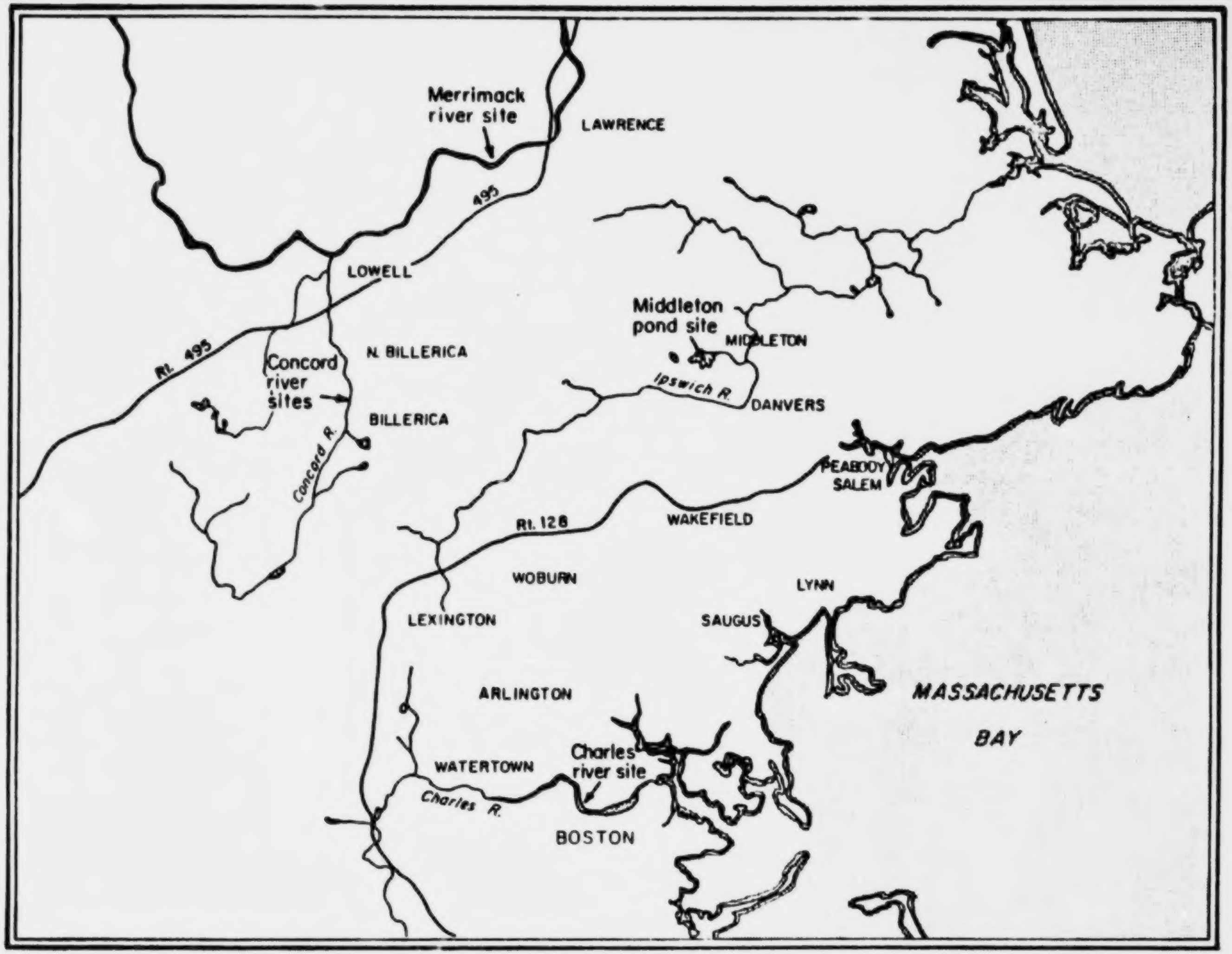

Merrimack river site
LAWRENCE
495
LOWELL
Rt. 495
N. BILLERICA
Concord river sites
BILLERICA
Concord R.
Middleton pond site
MIDDLETON
Ipswich R.
DANVERS
PEABODY
SALEM
Rt. 128
WAKEFIELD
WOBURN
LEXINGTON
LYNN
SAUGUS
MASSACHUSETTS
BAY
ARLINGTON
WATERTOWN
Charles river site
Charles R.
BOSTON

Figure 7. Massachusetts sampling sites.

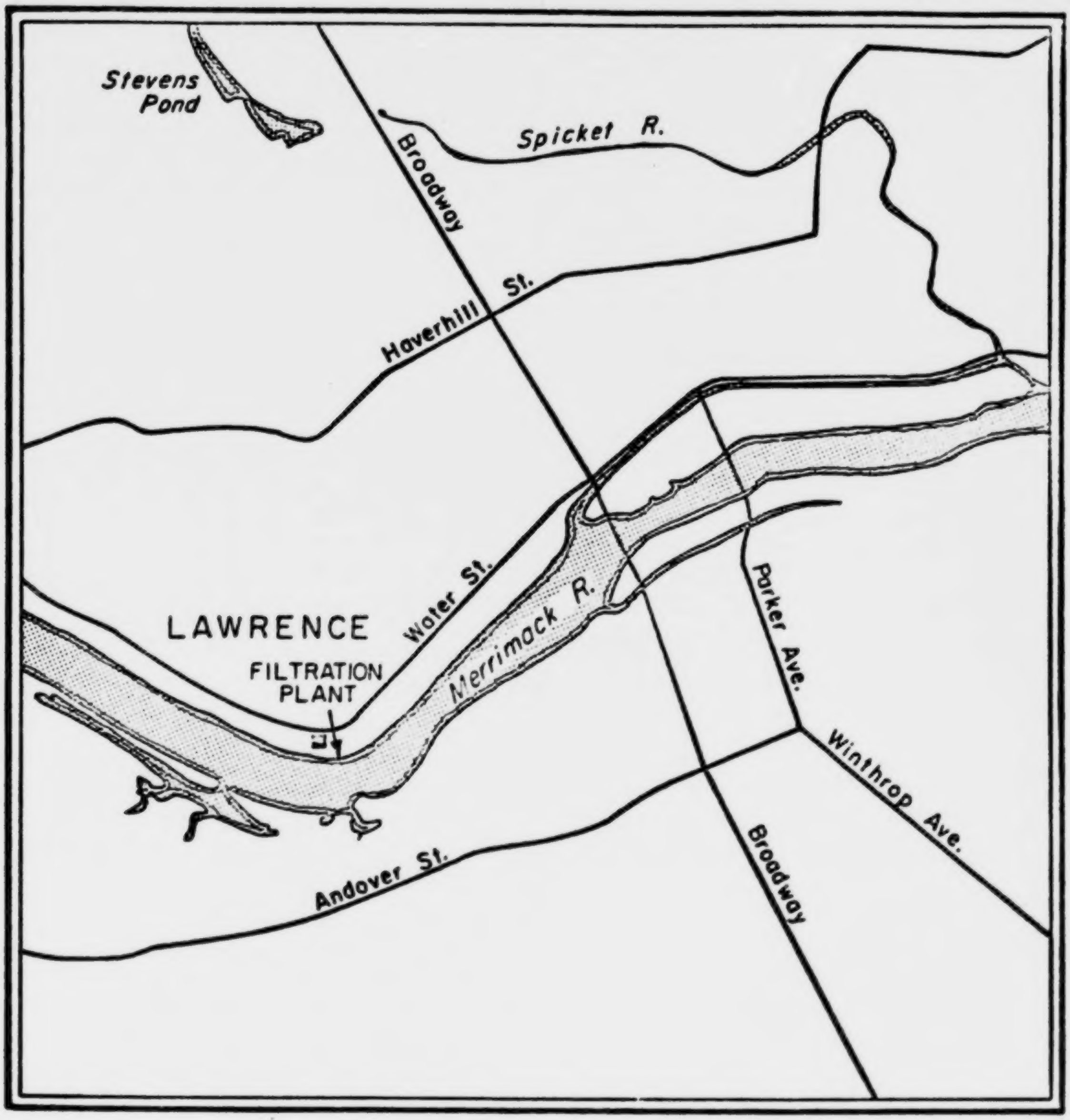

Stevens Pond
Broadway
Spicket R.
Haverhill St.
LAWRENCE
Water St.
FILTRATION PLANT
Merrimack R.
Parker Ave.
Winthrop Ave.
Andover St.
Broadway

Figure 8. Sampling site: Lawrence, MA.

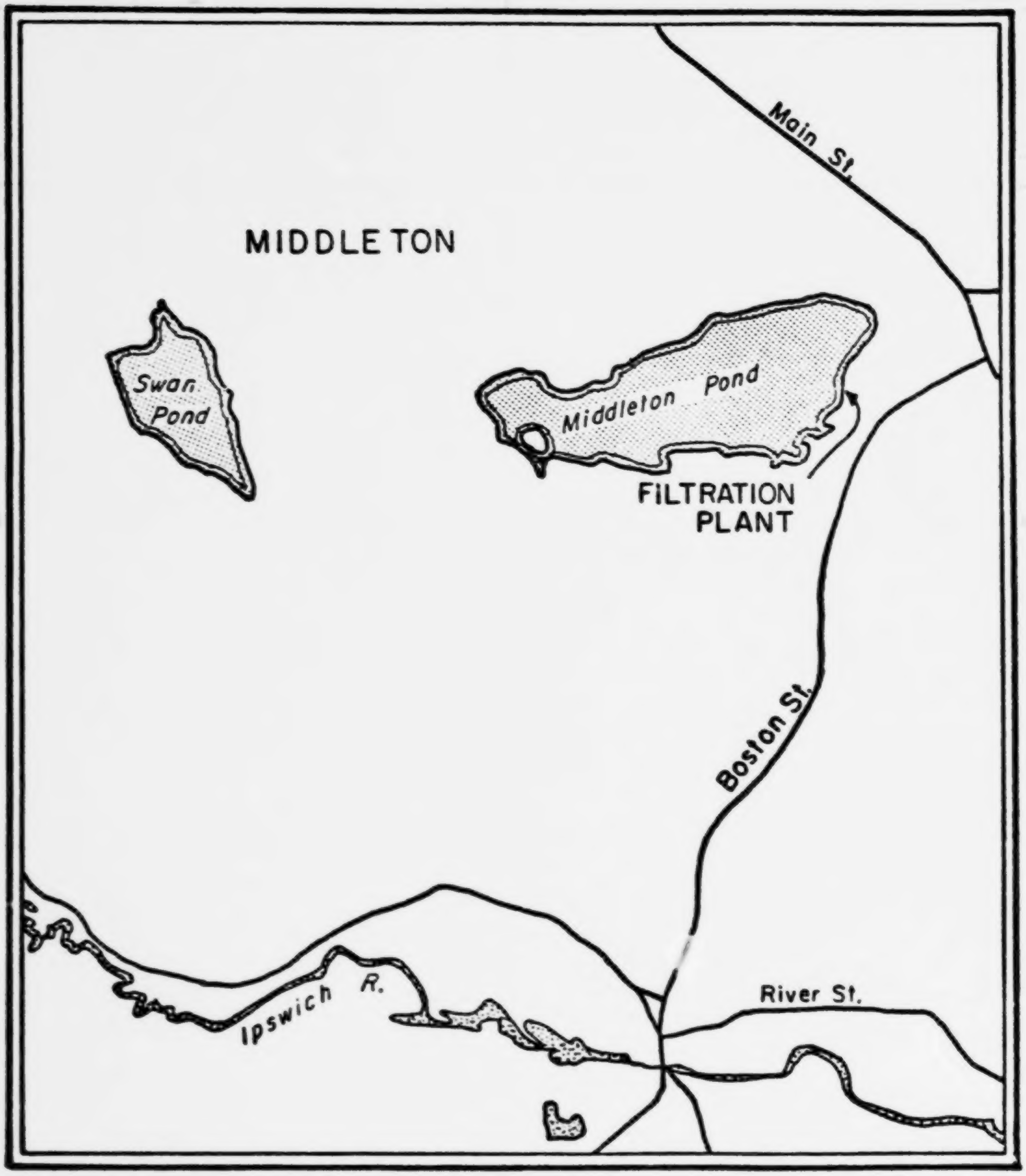

Main St.
MIDDLETON
Swan Pond
Middleton Pond
FILTRATION PLANT
Boston St.
River St.
Ipswich R.

Figure 9. Sampling site: Danvers, MA.

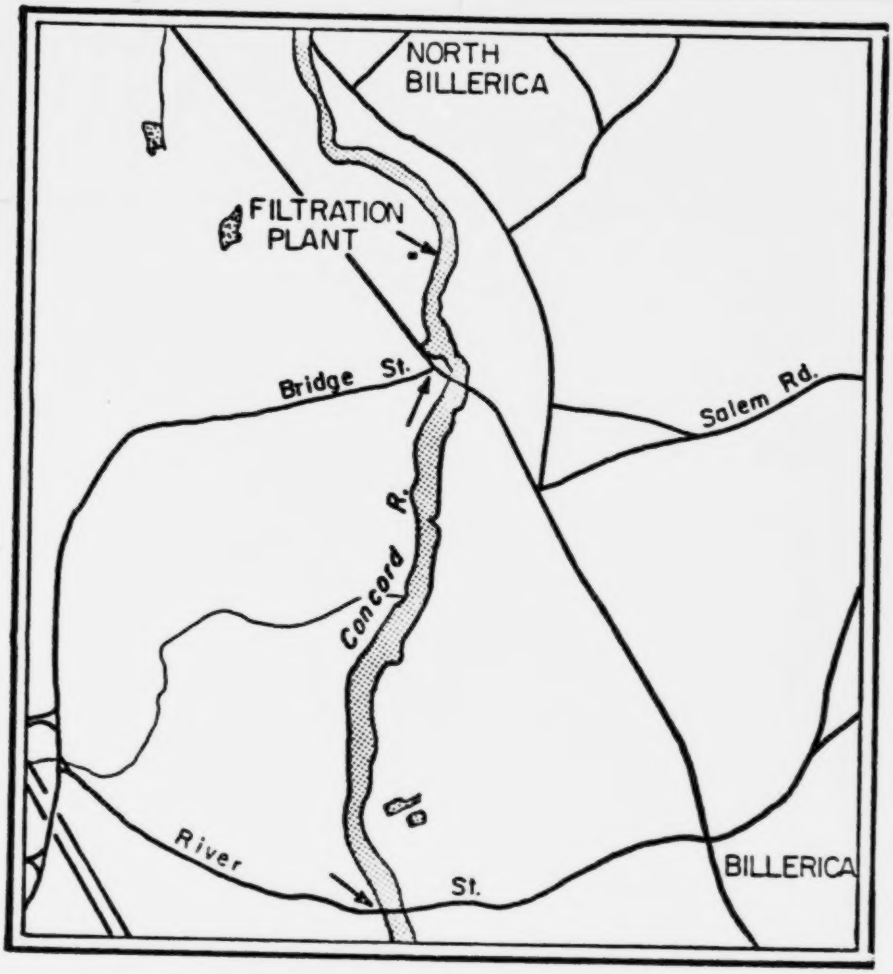


Figure 10. Sampling sites: Billerica, MA.

redissolved in chromatographic eluant, centrifuged, and frozen at -25°C for several weeks until chromatographic analysis was performed.

Filtration of Collected Samples

In the laboratory, the water samples were first filtered through Whatman #1 filter paper to remove suspended material greater than 10 μm in diameter. The filter paper was replaced whenever the filtration rate decreased appreciably because of clogging. Whatman filter papers GF/D (2.7 μm) and GF/F (0.7 μm) were also used for the second Danvers sample and with the filtration of the laboratory-grown algae cultures to remove suspended algal cells. Katz *et al.* (78) reported that only negligible U.V. absorbing material was lost by filtration through a 10 μm frit to remove suspended material.

Ammonia-N Determination

Ammonia-N was initially determined using the indophenol colorimetric reaction described by Dora Scheiner (79). A twenty-five milliliter filtered sample was allowed to react in a 50-ml flask with 10 ml of pH 12 buffer solution containing phenol and nitroprusside followed by dilution to 50-ml with hypochlorite reagent.

The buffer solution (pH 12) consisted of 30 g trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$), and 3g disodium ethylenediaminetetraacetate (EDTA) diluted to one liter with distilled ammonia-free water. The ammonia-free water was prepared by dosing distilled water with 1-2 mg/L chlorine and decomposing the active chlorine remaining after 6-8 hours with U.V. radiation. The phenol nitroprusside solution was made by dissolving 30g of phenol in a part of the pH 12 buffer solution followed by addition of 0.1 g sodium nitroprusside ($Na_2Fe(CN)_5NO \cdot 2H_2O$) and dilution to 500 ml with buffer solution. It was important to add the nitroprusside only after the phenol had been dissolved in the buffer solution to ensure a low reagent blank.

The alkaline hypochlorite reagent consisted of 15 ml of commercial bleach (Chlorox, 3.5% available chlorine) and 200 ml of sodium hydroxide solution diluted to 500 ml with distilled ammonia free water. Sodium hydroxide solution was made by dissolving 40g NaOH in distilled ammonia free water and diluting to one liter. Reagent grade sodium hypochlorite solution (Fisher Scientific Co., 4-6% NaOCl) was later used in place of the Chlorox to obtain lower reagent-blank values.

The sodium nitroprusside solution was prepared freshly every three weeks and stored at 4 degrees centigrade. Alkaline hypochlorite reagent was prepared daily when needed. All reagents were brought to room temperature prior to use.

After addition of the reagents to the water sample the flask was mixed well by inversion. Color development was considered complete after 45 minutes at room temperature. Absorbance was read at 635 nm against a reagent blank carried through the procedure along with each set of samples. A one cm cell was used to measure the absorbance of solutions having ammonia concentrations less than about 1 mg/L NH_3-N.

All glassware was acid washed in 10% HCl prior to use to remove trace ammoniacal impurities. A calibration curve was prepared by submitting samples containing known ammonia concentrations to the standard procedure. Stock ammonia solution (1,000 mg/L N; stable at least 6 months) was prepared by dissolving 3.810 g NH_4Cl, previously dried one hour at 100°C, in distilled ammonia-free water and diluting to one liter. Intermediate ammonia solutions (20 mg/L; stable 4 days) were made by diluting 2 ml stock ammonia solution to 100 ml with distilled, ammonia-free water. Standard ammonia solutions containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mg/L were prepared by dilution of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml of intermediate ammonia solution to 100 ml with distilled ammonia-free water. A Beckman double-beam spectrophotometer was used for the absorbance readings.

Because of difficulties in the reproducibility and color development in determining NH_3-N content after Kjeldahl digestion using Scheiner's method, ammonia-N was later determined using the scaled-down indophenol method of Strickland and Parsons (80). For this method phenol solution was prepared by dissolving 20 g crystalline analytical grade phenol in 200 ml of 95% ethyl alcohol. Sodium nitroprusside solution consisted of 1.0 g sodium nitroprusside in 200 ml ammonia-free water. The solution was stable for at least one month. Alkaline reagent was made by dissolving 100 g sodium citrate and 5 g analytical grade sodium hydroxide in 500 ml ammonia-free water. This solution was stable indefinitely. The oxidizing solution was prepared daily as needed and consisted of 10 ml reagent grade sodium hypochlorite diluted to 50 ml with alkaline stock solution.

Acid-washed 20-ml Pyrex test tubes were filled with ammonia-free water and capped with aluminum foil prior to use to lessen contamination by atmospheric ammonia. This water was discarded immediately prior to use for a determination. Five ml of sample were then pipetted into each test tube. The following solutions were sequentially added with automatic pipets and the samples were vortex mixed after each addition:

0.2 ml phenol solution;
0.2 ml nitroprusside solution;
0.5 ml oxidizing solution.

The test tubes were then capped with aluminum foil and allowed to stand at room temperature for one hour for complete color development. The color produced is said to be stable for at least 24 hours (80). Absorbance readings and preparation of calibration curves were done according to the previously described method.

Kjeldahl-Nitrogen Determination

Total Kjeldahl nitrogen was measured by ammonia determination after decomposition of the organically bound nitrogen in the minus three oxidation state in the water sample to ammonia by acid digestion. This method fails to account for the nitrogen in azides, azines, azo, hydrazones, nitrate, nitrite, nitrile, nitroso, oximes, and semicarbazones (44). Several digestion methods were used during the study. The digestion solution of Scheiner (79) which was employed initially, consisted of 134 g of potassium sulphate (K_2SO_4) dissolved in 650 ml of ammonia-free water and 200 ml of concentrated H_2SO_4. Five ml selenyl chloride ($SeOCl_2$) were added and the combined solution was diluted to one liter, after cooling, with ammonia-free water. Five ml of this digestion solution was added to a 25-ml water sample in a 100-ml Kjeldahl flask, and heated under a hood until all the water was removed. The residue was digested for a further 30 minutes and then cooled to room temperature. Small glass funnels were inserted into the mouths of the Kjeldahl flasks to help the digestion and prevent loss. After the sample had cooled, it was transferred to a 100-ml beaker by sequential rinsing with ammonia free water and neutralized to pH 7 or greater with 1 M NaOH. The neutralized sampled was then transferred to a 100-ml volumetric flask and filled to the mark with ammonia-free water. Twenty-five ml of the diluted neutralized sample was then analyzed according to Scheiner's ammonia method described previously.

Other digestion mixtures were employed with essentially the same procedure. These included:

(1) *Standard Methods digestion reagent* (44):
This is the same as Scheiner's reagent with the exception that 2 g of HgO is used as the catalyst per liter instead of 5 ml selenyl chloride.

(2) *Mague and Mague digestion reagent* (81):
0.2 g SeO_2 and 20 g K_2SO_4 were dissolved in 600 ml ammonia-free distilled water. 110 ml concentrated reagent grade H_2SO_4 was then added. After the mixture had cooled it was diluted to one liter with ammonia-free water.

(3) *Strickland and Parsons' digestion reagent* (80):
0.1 g of analytical, reagent-grade selenium dioxide (SeO_2) was dissolved in 500 ml ammonia-free distilled water. 500 ml concentrated, reagent-grade H_2SO_4 was then added. It was diluted to one liter with ammonia-free water after it had cooled.

Only 2 ml of the Strickland and Parsons' digestion solution was added to each water sample because of its stronger sulfuric acid content.

The most satisfactory results were obtained using a modified digestion solution of Strickland and Parsons' (80) followed by ammonia determination using the scaled-down indophenol method of Strickland and Parsons described previously. Twenty grams of K_2SO_4 was added to each liter of Strickland and Parsons digestion solution to raise the boiling point of the acid and help keep it refluxing close to the sample. Two ml of digestion solution was added to each 25-ml water sample in a Kjeldahl flask and digested for two hours following evaporation of the water. After they had cooled, the acid residues were each diluted with approximately 10 ml of ammonia-free water and transferred to 100-ml volumetric flasks. Sequential rinsing with additional ammonia-free water assured complete transfer. The diluted samples were then neutralized with 1 M NaOH to pH 6.0-7.6 after addition of one drop of bromothymol blue indicator. The solution was titrated to the bromothymol blue endpoint (yellow to faint blue) and then diluted to the mark with ammonia-free water. Five-ml aliquots were then analyzed for ammonia by the scaled-down indophenol method. The indophenol-blue color intensity was read against a reagent blank carried through the entire procedure because of the potential adsorption of atmospheric ammonia by the sulphuric acid during digestion.

Great care was required to prevent the contamination of reagents and samples by atmospheric ammonia or particulate ammonium salts. Solutions were kept in tightly stoppered bottles. All glassware was cleaned copiously with 10% HCl and rinsed thoroughly with ammonia-free water immediately prior to use. Kjeldahl flasks were initially cleaned by steeping them in near-boiling sulphuric acid for several hours. Ultra-pure sulfuric acid ('Ultrex,' J.T. Baker Co.) having a lot analysis of no more than 0.5 ppm ammonia was used in the digestion solution to ensure a low blank value.

Calibration curves were obtained with the standard ammonia solution described in Section entitled "Ammonia-N Determination". The calibration curve was stable, so that recalibration was not required for each batch of determinations, or even for new reagents carefully prepared. Organic nitrogen was calculated as the difference between the nitrogen found for digested (Kjeldahl) and undigested (NH_3) samples.

Resin Adsorption

After the water samples had been filtered through Whatman filter paper to remove suspended material, macroreticular resins were used to remove selectively potentially interfering hydrophobic carbonaceous compounds prior to concentration and chromatographic analysis of the remaining organic materials. The XAD macroreticular resins (Rohm and Haas Co., Philadelphia, PA) were thoroughly purified by sequential solvent extractions, of about 10 grams per batch, with methanol, acetonitrile and diethylether in a Soxhlet extractor for 8 hours per solvent. The purified resins were stored in glass stoppered bottles under methanol to maintain their high purity. Tenax (Applied Science Laboratories Inc., State College, PA) was purified with methanol and acetonitrile extractions while Ambersorb XE-340 was supplied already purified and hydrated with acetic acid solution from the manufacturer (Rohm and Haas Co.).

The purified resins were added as methanol slurries into 1 cm I.D. glass columns and were used either individually or as mixed resin beds. Single resin beds were prepared by addition of an individual resin to a depth of about 6 cm (1.5-2.0 grams dry resin). A glass wool plug was inserted near the stopcock of the column and above the resin bed. Mixed resin beds, consisted of equal volume combinations of either XAD-2 and XAD-4 or XAD-8 and Tenax-GC macroreticular resins, and were prepared by sequential addition of each resin to a depth of about 6 cm. Glass wool plus were inserted near the stopcock, between the two resin beds, and above the final resin layer.

The procedure for eluting compounds through the prepared column was the same for both the single and mixed resin beds. The methanol was drained to the top of the uppermost resin bed and then flushed with approximately 200 ml of distilled water. The distilled water was drained to the top of the resin bed prior to passage of a sample. A flow rate of about 10 ml/min was maintained by application of one psi pressure supplied from a regulated, filtered-air line. The first 20-ml of eluted sample was discarded because of the corresponding dead volume in the column containing the residual distilled water. The remaining eluted sample was collected for analysis. The resin was regenerated after elution of each liter of sample. Regeneration was initially achieved by equilibration with about 50 ml of methanol and diethylether for the XAD resins and 100 ml of methanol of the Tenax and XE-340 resins. The regenerating solvents were discarded. Later regeneration for the XAD-8 and Tenax resins was achieved by sequential equilibration with 50 ml of 10^{-2} M NaOH, 10^{-2} M HCl, and methanol followed by rinsing with 300 ml distilled water.

Field samples initially were passed through the resins without pH adjustment. Later samples were acidified to pH 2.0 with concentrated H_3PO_4 prior to passage through the resins.

Adsorption of commercially available humic acid on a combination of XAD-8 and Tenax resins at acid and basic pH values was investigated. The humic acid was dissolved in 1 liter of .03 M NaOH. Acidic humic acid solution was prepared by titration of about 980 ml of the basic humic acid solution to pH 2.0 with concentrated H_3PO_4 followed by dilution to one liter with ammonia-free water. Humic acid concentration was determined spectrophotometrically at 330 nm.

The ability of each macroreticular resin to adsorb selected carbonaceous substances or nitrogenous organic compounds was investigated by passage of known concentrations of reference compounds through individual resin beds, or a combination of them, at several pH values. Breakthrough curves for individual test compounds were determined by measuring their concentrations in the column effluents after passage of increasing volumes of sample through the resin. The filtered aliquots were then analyzed spectrophotometrically. U.V. absorbance was converted into concentration on the basis of standard curves of absorbance readings vs. known concentrations of the reference compound.

Breakthrough curves for mixtures of test compounds were also determined by measuring their concentrations in column effluents after passage of increasing volumes of sample through the resin. The filtered aliquots were first chromatographed to separate the mixtures into their constituent components. Concentrations of the separated compounds were obtained by integration of the areas of the resolved chromatographic peaks with a disc integrator.

Concentration

One to four liters of water previously filtered through Whatman filter paper and macroreticular resins was evaporated to approximately 200 ml using a Buchi, model R, flash evaporator. Evaporation was carried out below 30°C to avoid reaction and decomposition. The partially concentrated sample was then lyophilized using a VirTis automatic Freeze-Dryer (Model No. 10-010) to remove all remaining water. The residue was redissolved in the chromatographic eluant, and the resulting mixture centrifuged. The supernatent was then ready for chromatographic analysis. Concentrated samples were frozen at -25°C and stored for several weeks prior to analysis. Thawed samples were further centrifuged to remove suspended material immediately prior to chromatographic analysis.

Recovery values of total organic nitrogen were determined on samples concentrated by low temperature distillation and lyophilization using the scaled-down indophenol method of Strickland and Parson (80) described in Section 2. These values ranged from 11% for the non-XAD filtered Danvers bloom sample to 100% for the Concord River (6/8-78) and *Oscillatoria tenuis* resin filtered samples. A mean organic nitrogen recovery value of 51.7% (standard deviation = 29.2%) was observed for the field samples concentrated by this method.

Chromatographic Methods

Description of Chromatographic System--

The chromatographic system consisted of a DuPont 848 high-pressure pump with a DuPont 838 programmable gradient accessory, a Schoeffel (Westwood, NJ) SF 770 Spectroflow U.V. monitor with a SFA 339 wavelength drive assembly, a FS 970 spectrofluoro fluorescence monitor with a wavelength drive, a Duplex minipump (Laboratory Data Control, Riviera Beach, FL) and a Schoeffel MM 700 module.

The gradient accessory was used to increase gradually the strength of the mobile phase during a chromatographic separation. Gradient elution provided better resolution for a wider range of sample polarities than could be attained by conventional isocratic elution. The variable wavelength feature of the U.V. detector provided analytical flexibility to monitor compounds having maximum absorbance anywhere in the U.V. spectrum. Both the U.V. and fluorescence monitors were fitted with wavelength drive mechanisms for use in identifying unknown peaks by stopped-flow spectrum scanning. The memory module provided baseline correction during spectrum scanning and gradient programming. The minipump was used to deliver buffer and reagents for producing fluorescent derivatives to the column effluent before it entered the fluorometer.

Figure 11 is a schematic presentation of the chromatographic system. The fluorescence detector could be used without post-column derivatization to monitor naturally fluorescing compounds, or with post-column derivatization by introducing fluorescamine (4-phenylspiro-[furan-2(3H),1'-phthalan]-3,3'-dione) (Hoffman-LaRoche Inc., Nutley, NJ) and borate buffer prior to the fluorometer. These reagents react with primary amines to form fluorescent derivatives. All Teflon tubing, connectors and tees were purchased from Rainin Instrument Co. (Boston, MA). 250-ml reservoir bottles were placed approximately 3 feet above the minipump and connected to it by 1.5 mm I.D. × 3.0 mm O.D. teflon tubing. Air displacing the fluorescamine and borate buffer was first passed through 50% sulphuric acid in a gas trap to remove traces of primary amines. The exit valves of the pump were connected to 0.8 mm I.D. × 1.5 mm O.D. tubing. Coils A, B, and C were approximately 0.40 m, 1.5 m, and 9.0 m lengths of 0.3 mm I.D. × 1.5 mm O.D. tubing, respectively. The coiled pieces of tubing, A and B, were inserted to provide sufficient mixing of the reagents. Coil C was included to induce sufficient backpressure to prevent gas bubble formation when acetone was mixed with the aqueous buffer at the second tee. A back-pressure of about 40 psi was maintained and was monitored by two liquid pressure gauges. These gauges also helped to dampen the pulsating flow of reagents produced by the positive displacement reciprocating piston mechanism of the minipump.

Borate buffer was prepared by titrating 0.1 M boric acid to pH 9.3 with 4.5 M $LiOH \cdot H_2O$ and was introduced at a flow rate of about 15 ml/hour. The fluorescamine solution consisted of 15 mg/L in acetone and was used at a flow-rate calculated to be less than 25-30% of the total flow (equal to 15 ml/hour). The use of more concentrated fluorescamine solution has been reported in the literature (82). However, reagent precipitation in the lines tends to occur at such higher concentrations (83). Fluorescamine is stable at room

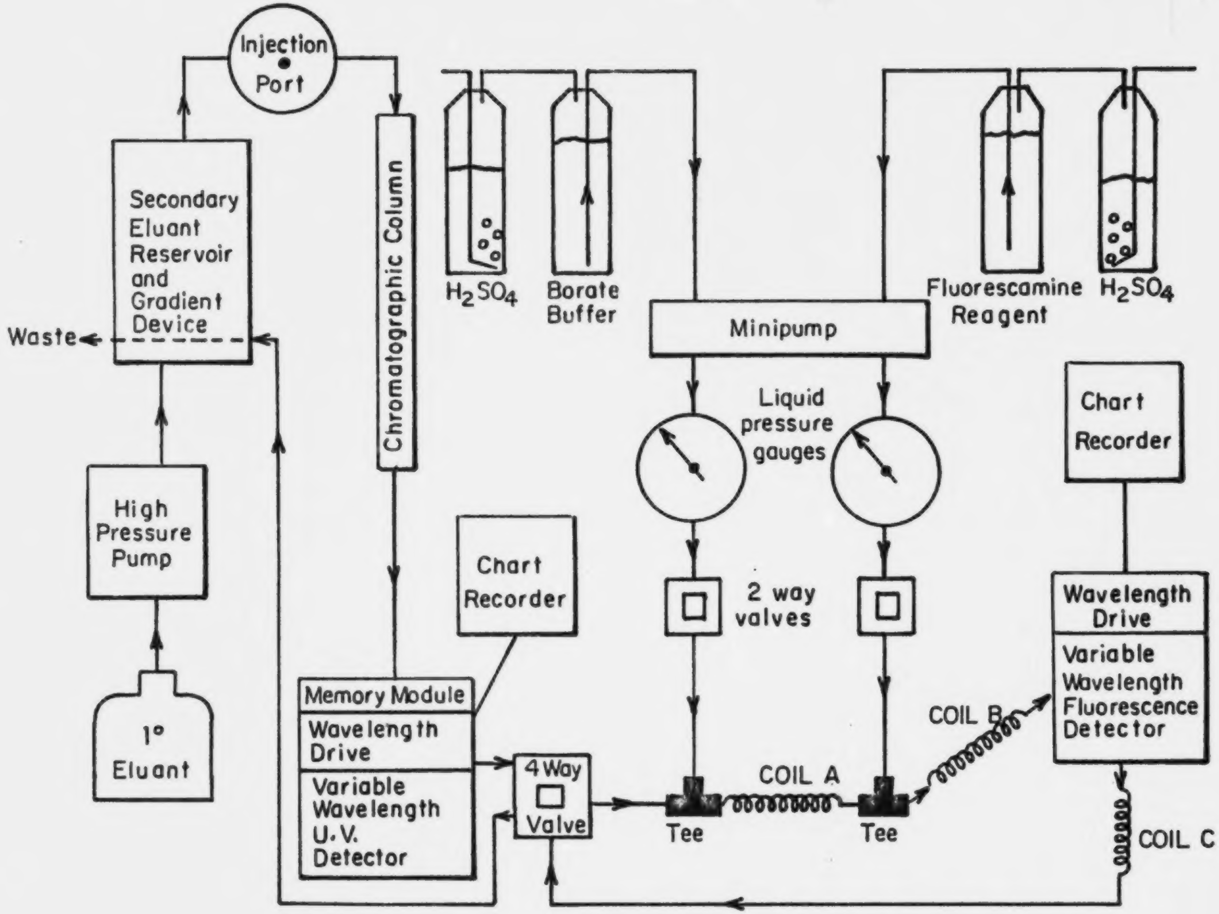

Injection Port
Secondary Eluant Reservoir and Gradient Device
Waste
High Pressure Pump
1° Eluant
Chromatographic Column
H_2SO_4
Borate Buffer
Minipump
Fluorescamine Reagent
H_2SO_4
Liquid pressure gauges
2 way valves
Chart Recorder
Memory Module
Wavelength Drive
Variable Wavelength U.V. Detector
4 Way Valve
Tee
COIL A
Tee
COIL B
Chart Recorder
Wavelength Drive
Variable Wavelength Fluorescence Detector
COIL C

Figure 11. Schematic diagram of chormatographic system.

temperature in both solution and powdered form (83,84). The acetone solution and borate buffer solution were made up only when the reservoirs were depleted. Before shut-down of the chromatographic system the tubing was flused with column eluant and borate buffer to avoid precipitation of fluorescamine in the lines. All glassware was acid washed in dilute HCl solution. Caution was taken to prevent contamination by traces of primary amine.

Early work was done using only the isocratic pump and U.V. detector without wavelength drive. It was found then difficult to achieve sharp resolution of constituent compounds and the analytical ability to identify unknown substances was limited severely. Later funding permitted the purchase of the additional chromatographic equipment described earlier in this section. Before purchase of the fluorometric equipment a preliminary study was undertaken to evaluate the ability of fluorescamine derivatization and fluorescence detection to monitor primary amines not detectable by conventional U.V. spectroscopy. Separate one to five ml portions were collected from the effluent of a chromatographic column after passage of a concentrated field sample. Two ml of eluate or diluted eluate was then allowed to react with 1 ml 0.2 M boric acid buffer (pH 9.5), 1 ml acetone and 0.2 ml fluorescamine solution with vortex mixing after addition of each reagent. The buffer was prepared, as suggested by the manufacturer of fluorescamine (Hoffman LaRoche, Inc.), by titrating 0.2 M boric acid with NaOH. Stein later recommended that LiOH be used in place of the NaOH when using the boric acid buffer in post column derivatization procedures, to help avoid precipitation of fluorescamine in the lines containing the mixed reagents. (Stein, S., personal communication, Roche Institute of Molecular Biology, Nutley, NJ 07110.) The fluorescamine solution was comprised of 100 mg fluorescamine per 100 ml acetone. The fluorescence intensity of derivatized sample was read immediately on a Turner, model 11 fluorescence meter using a Corning filter 5158 and a combination of filters 4-65 and 3-71 to set the excitation and emission wavelengths, respectively. Final fluorescence values were calculated from the differences between sample eluate and chromatographic eluant reagent blank.

Resolution of Injected Samples--

Injection volumes--Injection volumes from 5 to 50 µl of concentrated sample or reference compounds were introduced into the chromatographic system through a Rheodyne (Berkeley, CA) model 7120 syringe-loading injector. The reference standards, which were of the highest quality commercially available, were not purified further before use. The reference compounds were dissolved either in ammonia-free distilled water or in the chromatographic eluant. Guanine was dissolved in 0.14 M LiOH. The criterion used in determining the injection quantity was the volume required to produce maximum on-scale peak displacement and resolution on the U.V. and fluorescence chart recorders.

Other investigators have used larger injection volumes of 100 µl to 165 ml to achieve improved detection of trace contaminants (85,86). The maximum injection volume used in this study was limited to the capacity of the injection loop, 50 µl. Large-bore columns would also have been required to handle the increased load of a large injection volume.

Mobile phases and column supports--The different modes of chromatography studied to attain resolution of the largest number of nitrogenous compounds included: (1) cation exchange chromatography (Zipax SCX, DuPont Co.); (2) anion exchange chromatography (Aminex A-27, Bio-Rad Laboratories); (3) paired ion chromatography (Zorbax CN, DuPont Co.); (4) reversed phase chromatography (Zorbax C-8, Zorbax CN) and (5) normal phase chromatography (Corasil II, Waters Associates, or Zorbax CN). Concentrations of reagents used in making up sodium acetate-acetic acid; sodium citrate-citric acid; and ammonium acetate-acetic acid buffers at desired pH values and salt concentrations were determined from the equation

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\mathrm{salt}]}{[\mathrm{acid}]} \quad . \qquad (3)$$

0.05 M phosphate buffer (pH = 6.9) was made from equal molar concentrations of KH_2PO and Na_2HPO_4. Borax buffer was composed of 0.05 M sodium borate ($Na_2B_4O_7 \cdot 5H_2O$) adjusted with phosphoric acid.

Detection, Identification and Quantification of Resolved Constituents--

Eluted compounds were detected by U.V. and fluorescence spectroscopy with or without fluorescamine derivatization. Identification of unknown resolved chromatographic peaks was achieved by comparison of retention position and ultraviolet data with those of reference compounds. U.V. data was obtained by stopped-flow spectral scanning of individual chromatographic peaks. The memory model was used to store initially the background spectrum of the mobile phase, flow cell, and photomultiplier, and later subtract this background spectrum from that of the eluant to obtain the spectrum for the compound of interest. The resulting corrected spectra of reference compounds were then compared with corrected spectra of unknown chromatographic peaks. Positions of maximum and minimum UV absorbance and relative absorbance at several wavelengths were used as criteria for compound identification. Internal standards and fluorescence peaks resulting from fluorescamine responsive compounds were also used to identify unknown constituents. The internal standard consisted of a known quantity of reference compound which was thought to be present in the concentrated field sample. Sometimes the internal standard was added to the unknown solution before the chromatographic separation, while other times it was injected separately immediately before or after injection of the sample. When injected with the concentrated field sample, the suspect peak increased in proportion to the quantity of known reference compound injected. Either method compensated for errors made in the preparation of the eluting solution. The occurrence of a fluorescent peak indicated either the presence of a fluorescamine-response primary amine or a non-derivatized compound also fluorescing at the excitation and emission wavelengths used to detect fluorescamine-responsive compounds (390 nm excitation, 470 nm emission). The probability of identifying the unknown peak correctly increased with corroborative evidence from different identification methods.

Determination of peak area for some reference compounds was achieved with a series 200 disc integrator (Disc Instruments, Inc., Santa Anna, CA) attached to a potentiometric recorder. The integrator automatically computed peak area and displayed this computation as a second trace directly under each recorder peak. The number of integrator 'counts' was converted to concentration units by determination of the number of 'counts' of known quantities of reference compounds. Because of difficulties in maintaining the required baseline for proper use of the disc integrator amounts were later estimated by measurement of peak heights.

RESULTS

Introduction

A number of different analytical methods, isolation and concentration techniques, and chromatographic systems were used to obtain the results described in this section. Although the results of some methods proved to be unsatisfactory, the data are presented to give a complete picture for future investigators. The chromatographic results, both prior to and after the acquisition of the gradient pump and fluorescence equipment, are presented to illustrate the enhanced analytical ability resulting from the use of the more sophisticated equipment.

Retention of Organic Materials on Macroreticular Resins

Test Compounds--

The concentration and fractional recoveries of individual test compounds after passage of increasing sample volume through macroreticular resins are shown in Appendix A (Tables A-1 to A-23). Concentration values were calculated from least-square-fit equations determined from absorbance measurements of reference compounds at several concentrations (Appendix A, Table A-24). Recovery values were calculated according to the equation:

$$\%\ \text{recovery} = \frac{\text{effluent concentration of test compound}}{\text{influent concentration of test compound}} \times 100 . \qquad (4)$$

The percentage recoveries of constituents in mixtures of nitrogenous compounds after passage of increasing sample volumes through a combination of XAD-8 and Tenax resins are shown in Appendix A (Tables A-25 and A-26). Recovery values were calculated from concentration measurements determined either by peak height (H) or peak area (A) on chromatograms of the resolved mixture. The approximate volume of sample filtered at 80% and 90% recovery and the ultimate percentage recoveries for the individual compounds and mixtures of test compounds are summarized in Tables 5 and 6, respectively. 100% recovery of constituent compounds in a mixture containing: 5-chlorouracil, barbituric acid, thymine, guanine, creatinine, purine, pyrrole, adenine and tryptophan was observed after passage of only 70 ml through XAD-8 resin alone. Recovery values of constituent compounds in a mixture containing these nine nitrogenous materials after equilibrium for 15 minutes in various resin slurries are shown in Table 7.

Recovery values greater than 90% after passage of less than 100 ml of sample or after equilibration for 15 minutes in resin slurries occurred for most nitrogenous compounds using the XAD and/or Tenax resins under the indicated conditions. One noteworthy exception was the poor recovery of indole

TABLE 5. RECOVERY OF INDIVIDUALLY TESTED NITROGENOUS COMPOUNDS AFTER PASSAGE THROUGH MACRORETICULAR RESINS

Compound	Influent Concentration (mg/L)	Resin(s)	Conditions	mls to 80% breakthrough	mls to 90% breakthrough	Ultimate recovery (%)[a]	mls filtered at ultimate recovery	Adsorptive capacity of resin(s) for test compound[b] mg/g
Adenine	9.54	XAD-8 & Tenax	pH 2.5	50	80	98.95	300-350	0.16
Adenine	9.64	XAD-8 & Tenax	pH 7.1	60	80	100	300-350	0.19
5-Chlorouracil	9.69	XAD-8 & Tenax	pH 2.0	60	80	100	175-200	0.19
5-Chlorouracil	10.43	XAD-8 & Tenax	pH 7.01	45	65	100	350-400	0.16
Creatine	10.0	Tenax & XAD-8	pH unadjusted	80	95	99.17	266-286	0.27
Creatinine	3.19	XAD-2 & XAD-4	pH 2 with HCl	65	-	81	90-190	0.07
Cytosine	8.56	XAD-2 & XAD-4	pH unadjusted	75	-	88	175-200	0.21
Cytosine	8.35	XAD-2 & XAD-4	pH 3 with HNO_3	40	165	90	165-190	0.11
Cytosine	10.0	Tenax & XAD-8	pH unadjusted	71	91	96	206-287	0.24
Humic Acid	13.48	XAD-8 & Tenax	pH 2	-	-	36.0	475-545	-
Humic Acid	18.34	XAD-8 & Tenax	pH 11.7	120	-	87.9	175-200	-
Indole	8.74	XAD-2 & XAD-4	10^{-3} NaOH	-	-	10.3	125-215	-

(continued)

TABLE 5 (continued)

Compound	Influent Concentration (mg/L)	Resin(s)	Conditions	mls to 80% break-through	mls to 90% break-through	Ultimate recovery (%)[a]	mls filtered at ultimate recovery	Adsorptive capacity of resin(s) for test compound[b] mg/g
Indole	6.57	XAD-4	10^{-3} HNO_3	-	-	4.8	270-370	-
Purine	10.00	XAD-8 & Tenax	pH 2.0	35	60	98.18	150-175	0.12
Purine	10.24	XAD-8 & Tenax	pH 7.0	30	40	100	175-200	0.10
Pyrimidine	10.0	XAD-8 & Tenax	pH 2.0	70	175	94.62	250-300	0.23
Pyrimidine	9.75	XAD-8 & Tenax	pH 7.0	50	65	99.32	350-400	0.16
Succinimide	30.00	XAD-8 & Tenax	pH 2.0	175	250	93.00	350-400	1.75
Succinimide	30.00	XAD-8 & Tenax	pH 7.05	60	125	93.15	175-200	0.60
Tryptophan	9.66	XAD-8 & Tenax	pH 2.0	150	300	92.52	350-400	0.48
Tryptophan	10.0	XAD-8 & Tenax	pH 6.9	35	45	100.00	350-400	0.12
Uracil	10.0	XAD-8	pH unadjusted	56	72	98	147-322	0.37

[a] % Recovery = (maximum effluent concentration/influent concentration) x 100

[b] Adsorptive capacity calculated at 80% recovery (for 3 g combined resin)

TABLE 6. RECOVERY OF MIXTURE OF NITROGENOUS COMPOUNDS AFTER PASSAGE THROUGH XAD-8 AND TENAX RESINS (3 GRAMS TOTAL RESIN)

compound	condition	concentration (mg/L)	mls to 80% breakthrough	mls to 90% breakthrough	ultimate recovery (%)[a]	mls filtered at ultimate recovery	adsorptive capacity of resins for test compounds (mg/g)[b]
uracil		5	45	100	100	200	.08
indole		5	-	-	0	400	-
tyrosine		10	100	200	100	300	.34
purine	pH of mixture unadjusted	10	100	100	100	200	.34
guanine		15	200	300	91.4	300	1.00
cytosine		40	100	150	100	300	1.33
adenine		20	150	200	100	300	1.00
creatinine		50	100	200	100	300	1.67
tryptophan		20	100	100	100	300	0.67
uracil		5.0	100	150	100	300	.17
indole		5.0	-	-	-	500	-
tyrosine	pH of mixture adjusted to pH 2.0 with HCl	10.0	100	150	100	150	.33
purine		10.0	75	150	100	300	.25
guanine		15.0					.37
cytosine		40.0	50	50	100	300	0.67
adenine		20.0	100	100	100	300	0.67
creatinine		50.0	75	75	100	300	1.25
tryptophan		20.0	200	200	100	200	1.33

[a] % recovery = (maximum effluent concentration/influent concentration) x 100

[b] adsorptive capacity calculated at 80% recovery.

TABLE 7. RECOVERY OF MIXTURE OF NITROGENOUS COMPOUNDS AFTER EQUILIBRATION FOR 15 MINUTES IN RESIN SLURRY (pH UNADJUSTED)[a]

Compound	recovery from XE slurry	recovery from Tenax slurry[b]	recovery from XAD-8 slurry
5-chlorouracil	35	88-100	100
barbituric acid	75	100	100
thymine	39	98-100	84-100
guanine	0	100	100
creatinine	25	100	85-100
purine	0	93-100	63- 89
pyrrole	0	95-100	-
adenine	13	95-100	49- 68
tryptophan	42	79-100	72- 95

[a] Upper percentage numbers are maximum recovery values computed from the highest observed concentration of compound after equilibration with resin slurry divided by the lowest observed concentration value of compound determined before equilibration with resin slurry.

$$\%\ \text{recovery} = \left(\frac{\text{concentration of compound after equilibration with resin slurry}}{\text{concentration of compound before equilibration with resin slurry}}\right) \times 100$$

[b] Single or initial numbers are mean values.

after passage through all the resins tested. Most nitrogenous compounds were strongly adsorbed onto XE resin and, therefore, poor recovery values were observed. No significant differences were observed for the recovery values of nitrogenous compounds through different resins with or without pH adjustment. Humic acid, however, was more strongly adsorbed onto a combination of XAD-8 and Tenax resins at pH 2.0 than at pH 11.7.

The variability in recovery values obtained from different chromatograms of resolved mixtures of nitrogenous compounds (Appendix A, Tables A-25 and A-26) is partially attributable to the difficulty in maintaining the baseline on the potentiometric chart recorder at zero, as is required for proper operation of the disc integrator. Baseline fluctuation in excess of the 3% recommended for maximum drift correction was frequently encountered, making integration difficult and inexact. This problem may also have contributed to the somewhat greater recovery values observed in these mixtures in comparison with the recovery values obtained with individually tested compounds.

The adsorptive capacities of the resins for the several tested compounds are shown in Tables 5 and 6. These values were calculated from the equation:

$$\underset{\text{(mg compound/gram resin)}}{\text{Adsorptive capacity}} = \frac{\text{influent concentration} \times \text{liters filtered to reach 80\% recovery}}{\text{grans resin used}} , \quad (5)$$

and represent the maximum quantity of compound adsorbed per weight of resin. Quantities of compounds exceeding the adsorptive capacities will break through into the column effluent. The adsorptive capacity of the resins (with the exception of indole) for the N-organic compounds ranged from only 0.08 to 1.75 mg per gram of resin as compared to the greater adsorption (5-25 mg carbon per gram resin) of non-nitrogenous compounds reported in the literature (87,88). In addition, the similar values obtained from filtered mixtures of N-organic compounds and individually tested materials indicate a non-competitive adsorption phenomenon.

NH_3-N and Kjeldahl-N--

Adsorption of NH_3-N and total Kjeldahl-N onto the macroreticular resins was also examined. Table 8 summarizes the percentages of NH_3-N, Kjeldahl-N, and organic-N found to pass through the macroreticular resins under study. The mean percentage recoveries for NH_3-N, Kjeldahl-N, and organic-N, for the different filtration conditions and their standard deviation are shown in Table 9. Statistical tests (Table 9) showed that the observed differences in recoveries were not attributable to the different resins and conditions used within the indicated confidence intervals. Over 93% of the NH -N and 88% of the organic-N contained in the field and laboratory samples were not adsorbed onto the individual resins or combinations of resins tested.

Kjeldahl-N and NH_3-N Determination

Concentration vs. Absorbance Data--

Concentration vs. absorbance readings at 635 nm using Scheiner's (79) method and the scaled down procedure of Strickland and Parsons (80) are shown in Tables 10 and 11, respectively. The following least squares linear equations were derived from these data and were used to calculate unknown Kjeldahl-N and NH_3-N concentrations in field and laboratory samples from U.V. absorbance readings. The equations were constrained to pass through the origin.

$$NH_3\text{-N[mg/L]} = 1.265\text{[(mg/L)/AU]} \times \text{(absorbance at 635 nm)[AU]} \quad (6)$$
$$\text{(Scheiner's method)}$$

$$\text{Kjeldahl-N[mg/L]} = 1.475\text{[(mg/L)/AU]} \times \text{(absorbance at 635 nm)[AU]} \quad (7)$$
$$\text{(Scheiner's method)}$$

$$NH_3\text{-N and Kjeldahl-N[mg/L]} = 1.070\text{[(mg/L)/AU]} \times \text{(absorbance at 635 nm)[AU]} \quad (9)$$
$$\text{(Strickland and Parsons' method)}$$

TABLE 8. PERCENTAGES OF NITROGENOUS MATERIAL PASSING THROUGH MACRORETICULAR RESINS[a]

date	method of NH_3-N determination & pH conditions[b]	source[c]	resin	% recoveries		
				NH_3-N	Kjeldahl-N	organic-N
11/15/77	N-1	Charles River	XAD-4 & XAD-2	99	87	78
12/1/77	N-1	Spy Pond	XAD-4 & XAD-2	100	100	100
12/16/77	N-1	Charles River	XAD-8	98	95	94
1/30/78	N-1	Charles River	XAD-8	100	100	100
3/2/78	N-1	Billerica WTP influent	XAD-8	100	100	100
3/2/78	N-1	Billerica WTP effluent	XAD-8	100	100	100
3/2/78	N-1	Lawrence WTP influent	XAD-8	81	100	100
3/2/78	N-1	Lawrence WTP effluent	XAD-8	96	52	48
3/9/78	N-1	Marlboro West WW pre Cl_2	XAD-8	98	93	89
3/9/78	N-1	Marlboro West WW post Cl_2	XAD-8	99	100	100
5/3/78	N-1	Marlboro West WW pre Cl_2	XAD-8 & Tenax	89	75	62

(continued)

TABLE 8 (continued)

date	method of NH_3-N determination & pH conditions[b]	source[c]	resin	% recoveries		
				NH_3-N	Kjeldahl-N	organic-N
6/9/78	N-2	Billerica water supply	XAD-8 & Tenax	92	100	100
6/14/78	N-2	Lawrence water supply	XAD-8 & Tenax			
		(first 100 ml)		81	84	86
		(100-1,000 ml)		93	97	98
6/21/78	N-2	Danvers water supply	XAD-8 & Tenax			
		(first 100 ml)		0	66	67
		(100-1,000 ml)		100	100	98
6/21/78	A-2	Danvers water supply	XAD-8 & Tenax			
		(first 100 ml)		91	76	78
		(100-1,000 ml)		-	83	83
6/23/78	A-2	Ohio supply	XAD-8 & Tenax			
		(first 100 ml)		100	84	82
		(100-1,000 ml)		35	87	91
incubation day 44	A-2	flask #2 *Oscillatoria tenuis*	XAD-8 & Tenax			
		(first 100 ml)		100	37	35
		(100-1,000 ml)		68	73	73
incubation day 43	A-2	flask #2 *Anabaena flos aquae*	XAD-8 & Tenax	100	72	71

(continued)

TABLE 8 (continued)

date	method of NH_3-N determination & pH conditions [b]	source [c]	resin	% recoveries		
				NH_3-N	Kjeldahl-N	organic-N
7/4/78	A-2	Billerica: Bridge Street	XAD-8 & Tenax	76	81	82
7/11/78	A-2	Danvers bloom (filtered to 0.62 μm)	XAD-8 & Tenax	94	98	98

[a] Samples eluted through approximately 3 g (dry weight) resin or combination of resins. % recoveries were determined after elution of 1 liter of sample through the individual resin or resin combination.

[b] N = influent pH value unadjusted
A = influent pH adjusted to 2.0 with H_3PO_4
1 = NH_3 determined using Sheiner's method
2 = NH_3 determined using Strickland and Parsons' method

[c] WTP = water treatment plant
WW = waste water
pre Cl_2 = pre chlorinated
post Cl_2 = post chlorinated

TABLE 9. MEAN PERCENTAGE RECOVERIES OF NH_3-N, KJELDAHL-N, AND ORGANIC-N THROUGH MACRORETICULAR RESINS

resins	influent pH value	mean percent recovery (standard deviation)			N[a]	t-statistic[b] for organic N recovery	P(|t|>computed t value) (two tailed test)[c]	F statistic for organic N recovery[d]	accept or reject null hypothesis[e]
		NH_3-N	Kjeldahl-N	organic-N					
XAD-8 & Tenax	pH 2	86 (13)	82 (10)	83 (10)	6	-0.65 (8)	53%	3.25 (6.23)	accept
XAD-8 & Tenax	unadjusted (neutral)	94 (4)	93 (12)	90 (18)	4	-0.12 (12)	91%	1.23 (4.47)	accept
XAD-2 & XAD-4 or XAD-8 alone	unadjusted (neutral)	97 (6)	93 (15)	91 (17)	10	0.42	68%	1.23 (2.77)	accept
overall average for all resins & conditions tested	-	94 (9)	90 (13)	88 (15)	20	-	-	-	-

[a] N = sample size

[b] $t = (X_1 - X_2)/(S_1^2/n_1 + S_2^2/n_2)^{1/2}$; where degrees of freedom are indicated in parentheses ($= n_1 + n_2 - 2$); t test performed on recovery values for conditions shown in successive line of table; S^2 = variance.

[c] P(|t|>computed t value): expected frequency of observing given difference in means assuming both sample means taken from same population.

[d] $S_1^2/S_2^2 = \frac{\sigma_1^2}{\sigma_1^2} F(N_1 - 1, N_2 - 1)$; number in parenthesis is the 5% level (two tailed) of the distribution of F

[e] Null hypothesis: $\sigma_1^2 = \sigma_2^2$, i.e., sample variance taken from same population

TABLE 10. CONCENTRATION vs. ABSORBANCE READINGS[a] AT 635 nm (1 cm PATHLENCTH)

determination	concentration of standard (mg/L)	absorbance at 635 nm
NH_3-N	0.10	.095
	0.10	.101
	0.20	.147
	0.40	.307
	0.60	.463
	0.80	.635
	0.80	.639
Kjeldahl-N	0.10	0.026
	0.10	0.026
	0.20	0.105
	0.20	0.103
	0.40	0.260
	0.40	0.265
	0.60	0.422
	0.60	0.423

[a] determined by Scheiner's method (79).

TABLE 11.

NH_3-N CONCENTRATION vs. ABSORBANCE AT 635 nm DETERMINED BY STRICKLAND AND PARSONS' (80)
INDOPHENOL-HYPOCHLORITE METHOD

concentration (mg/L)	replicates of absorbance at 635 nm[a]						mean	standard deviation
	a	b	c	d	e	f		
0.10	0.104	0.099	0.100	0.094	0.080	0.086	0.094	.009
0.30	0.294	0.277	0.275	0.287	0.287	0.245	0.278	.017
0.50	0.450	0.472	0.485	0.500	0.440	0.458	0.468	.023

[a] 1 cm cell

where

$$AU = \text{absorbance units} = -\log \frac{I}{I_o} \quad .$$

Strickland and Parsons' scaled down method was used in later work of this research because of its excellent reproducibility (9.5%, 6.1%, and 4.9% standard deviations at 0.1, 0.3, and 0.5 mg/L NH_3-N, respectively), and ease in analyzing replicate samples. Reagent blanks were carried throughout all procedures because of increased absorbance attributable to ambient NH_3-N contamination. Typical NH_3-N reagent blank absorbance readings (635 nm), after Kjeldahl digestion using different digestion solutions and durations are shown in Appendix A (Table A-28). All the digestion conditions yielded similarly low reagent blank values with good reproducibility. A mean of 0.149 AU and standard deviation equal to 0.014 AU (9.5%) were observed for replicate reagent blank absorbance values of digestion solution D read against a dstilled water reference. Mean NH_3-N reagent blank absorbance readings (635 nm) without digestion were 0.050 mg/L and 0.04 mg/L, using reagent grade sodium hypochlorite solution and commercial Chlorox bleach, respectively. The calculated mean reagent blank absorbance value for digestion solution D with an undigested NH_3-N reagent blank reference was, therefore, 0.105 AU, or 0.11 mg/L NH_3-N. The blank values were most likely attributable to absorption of atmospheric ammonia by sulfuric acid.

Kjeldahl Digestion Solutions--

Table A-29 in Appendix A shows Kjeldahl-N concentration values for several field samples obtained after digestion with sulfuric acid solutions containing different catalysts for several periods of time. $SeOCl_2$ and SeO_2 as catalysts yielded similar, consistent, results after 1, 2 or 3 hours of digestion. One very poor recovery of Kjeldahl-N was observed using HgO as catalyst.

Field and Laboratory Samples--

Table 12 shows Kjeldahl-N, NH_3-N and organic-N concentrations of field and laboratory samples. Organic nitrogen was computed as the difference between nitrogen values after digestion (Kjeldahl-N) and before digestion. Organic nitrogen comprised 65-99% of the total Kjeldahl-N of the water supply samples (mean = 84%, standard deviation = 14%), and ranged from 0.30 - 21.7 mg/L. The unusually high organic-N content in the second Danvers water supply sample was attributable to the extracellular products of a blue-green algal bloom that was occurring at the time of sampling. The possibility that algal cell materials rather than extracellular products were contributing to this high organic-N value was investigated by measuring the organic-N content after further filtration to 0.7 μm. Although the bulk of residual green color was removed by the ultrafiltration, no decrease in organic-N or NH_3-N was observed. The mean 'non-bloom' organic-N content and standard deviation for the water supplies were 0.76 mg/L and 0.24 mg/L, respectively.

Concentration Efficiencies

By concentration efficiency is meant the percentage of original organic-nitrogen found still present after concentration. Concentration efficiencies for field samples and test compounds were calculated from Kjeldahl-N and U.V.

TABLE 12. NH_3-N AND KJELDAHL-N DETERMINATIONS

			non-resin filtered		non-resin filtered		resin filtered		organic nitrogen	
date	pH value & NH_3-N method[a]	source[b]	Kjeld-N (mg/L)	NH_3-N (mg/L)	% organic-N	% NH_3-N	Kjeld-N (mg/L)	NH_3-N (mg/L)	non-resin filtered mg/L	resin filtered mg/L
11/15/77	N1	Charles River	1.9	0.8	60	40	1.7	0.8	1.2	0.9
12/1/77	N1	Spy Pond	0.8	0.3	66	34	0.8	0.3	0.5	0.6
12/16/77	N1	Charles River	7.3	1.4	81	19	6.9	1.4	5.9	5.5
1/30/78	N1	Charles River	5.1	1.8	66	34	5.3	1.8	3.4	3.5
3/2/78	N1	Billerica WTP inf.	0.9	0.3	65	35	2.7	0.5	0.6	2.2
3/2/78	N1	Billerica WTP eff.	0.7	0.1	91	9	1.1	0.1	0.6	1.1
3/2/78	N1	Lawrence WTP inf.	1.5	0.5	68	32	1.8	0.4	1.1	1.4
3/2/78	N1	Lawrence WTP eff.	1.3	0.1	92	8	0.7	0.1	1.2	0.6
3/9/78	N1	Marlboro E. WW pre Cl_2	0.1	0.1	0	100	0.1	0.1	0	0
3/9/78	N1	Marlboro W. WW pre Cl_2	4.4	1.8	60	40	4.0	1.7	2.6	2.3
3/9/78	N1	Marlboro W. WW post Cl_2	4.3	2.1	52	48	4.4	2.1	2.2	2.2
6/9/78	N2	Billerica water supply	0.4	0.1	69	31	0.5	0.1	0.3	0.3
6/14/78	N2	Lawrence water supply								
		(first 100 ml)	0.8	0.2	75	25	0.6	0.2	0.6	0.5
		(100-1,000 ml)	-	-	-	-	0.7	0.2	0.6	0.6
6/21/78	N2	Danvers water supply								
		(first 100 ml)	0.7	0.1	99	1	0.5	0	0.7	0.5
		(100-1,000 ml)	-	-	-	-	0.7	0.1	0.7	0.7

(continued)

TABLE 12 (continued)

			non-resin filtered		non-resin filtered		resin filtered		organic nitrogen	
date	pH value & NH_3-N method[a]	source[b]	Kjeld-N (mg/L)	NH_3-N (mg/L)	% organic-N	% NH_3-N	Kjeld-N (mg/L)	NH_3-N (mg/L)	non-resin filtered mg/L	resin filtered mg/L
6/21/78	A2	Danvers water supply (first 100 ml)	0.7	0.1	98	2	0.5	0.1	0.7	0.5
		(100-1,000 ml)	-	-	-	-	0.6	0.1	0.7	0.6
-	-	humic acid (19.9 mg/L)	0.5	0.1	91	9	-	-	0.4	-
-	-	humic acid (19.9 mg/L replicate	0.4	0.1	82	19	-	-	0.3	-
6/23/78	A2	Ohio supply (first 100 ml)	0.6	0.1	92	8	0.5	0.1	0.5	0.5
		(100-1,000 ml)	-	-	-	-	0.5	0.1	0.5	0.5
incubation day: 44	A2	flask #2 *Oscillatoria tenuis* (first 100 ml)	1.8	0.1	97	3	0.7	0.1	1.8	0.6
		(100-1,000 ml)	-	-	-	-	1.3	0.1	1.8	1.3
incubation day: 43	A2	flask #2 *Anabaena flos aquae*	2.3	0.1	98	2	1.7	0.4	2.2	1.6
7/4/78	A2	Billerica (River Street)	1.6	0.1	92	8	-	-	1.5	-
7/4/78	A2	Billerica (Bridge Street)	2.8	1.0	65	35	2.2	0.7	1.8	1.5
7/11/78	A2	Danvers bloom filtered to 0.62 μm)	22.0	0.3	99	1	21.5	0.3	22.0	21.2

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[a] N = influent pH value unadjusted
A = influent pH adjusted to 2.0 with H_3PO_4
1 = NH_3 determined using Sheiner's method (79)
2 = NH_3 determined using Strickland and Parsons' method (80)

[b] WTP = water treatment plant
WW = waste water
pre Cl_2 = pre chlorinated
post Cl_2 = post chlorinated
inf. = influent
eff. = effluent

absorbance measurements, respectively. Table 13 summarizes the organic nitrogen recovery values of field samples subjected to low temperature rotary evaporation from a pear shaped flask followed by lyophilization and dissolution in chromatographic mobile phase solvent. Recovery values of field samples subjected to this method were variable, raging from 11% to 100% and having a mean of 51% and a standard deviation of 29%. Incomplete recovery of organic nitrogen in these samples was attributed to: incomplete transfer of lyophilized materials, and inability to redissolve all the lyophilized material in the small volume of solvent required to achieve a 1,000 to 2,000 fold concentration factor. Recovery values greater than 100% for some samples was attributed to the presence of small quantities of particulate or colloidal materials in the concentrated sample. It is thought that the presence of these undissolved materials during acid digestion resulted in increased organic nitrogen values in the digested solution.

The lowest recovery values were observed in the raw and resin-filtered Middleton Pond sample (11% and 13%, respectively), which had been collected during the occurrence of a blue-green algae bloom. The amount of lyophilized material, in the raw and filtered sample was substantially greater than for other sites. This was partially attributed to the comparatively greater level or organic nitrogen (equal to about 21 mg/L) in the sample. It was therefore hypothesized that the inability to completely dissolve this larger amount of lyophilized material in the required small volume of solvent resulted in the lower recovery values.

The mean recovery value for the field and laboratory samples, excluding the Middleton Pond site just discussed, was equal to 60% (standard deviation = 24%). This represented a somewhat greater value for the recovery of nitrogenous organic material than obtained using the low temperature evaporation procedure previously described.

Chromatographic Resolution of Reference Compounds

Initial Studies: Isocratic Elution--

A number of liquid chromatographic conditions were tested for their ability to resolve mixtures of known nitrogenous compounds. The results are shown in Appendix A (Tables A-32 to A-36). Attempts to separate nitrogenous mixtures on Aminex A-27 using 0.325 M ammonium acetate eluant, on Zipax SCX using perchloric, phosphoric, acetic and nitric acid mobile phases of varying strength, and on Zorbax CN using sodium-acetate-acetic-acid buffer with varying acetonitrile or methanol composition proved unsuccessful. Separation was later achieved on the Zipax column using mobile phases comprised of either KH_2PO_4, K_2HPO_4, NaH_2PO_4, or $NH_4H_2PO_4$ at different molar concentrations and adjusted to pH values of 2.5, 3.5, or 4.5 (Tables 14 to 16). Resolution of the larges number of compounds on this column was achieved using NaH_2PO_4 or $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4. Compounds were eluted more rapidly using eluants having greater salt concentrations, and at more elevated temperatures. Acetonitrile modifier did not improve resolution. Additional column length increased retention time but did not improve resolution of rapidly eluting compounds. Elution with a weaker mobile phase (distilled water adjusted to pH 2.5 with H_3PO_4) did not improve the resolution of this group of

TABLE 13. CONCENTRATION EFFICIENCY: ROTARY EVAPORATION IN PEAR-SHAPED FLASK FOLLOWED BY LYOPHILIZATION AND DISSOLUTION IN CHROMATOGRAPHIC MOBILE PHASE SOLVENT

sample	organic-N before concentration (mg/L)	concentration factor	calculated organic-N value after concentration[a] (mg/L)	observed organic-N value after concentration (mg/L)	concentration efficiency[b] (%)
Concord River, Billerica, MA 6/8/78[c]	0.3	2,000	671	965	>100
Merrimack River, Lawrence, MA, 6/14/78[c]	0.6	2,000	1,182	755	64
Middleton Pond, Danvers, MA 6/21/78[c]	0.6	2,000	1,130	582	52
Oscillatoria tenuis					
flask #2[c]	0.6	1,630	1,006	1,157	>100
flask #3[d]	1.1	1,800	2,030	983	48
Anabaena flos aquae					
flask #2[c]	2.2	1,615	3,621	1,236	35
flask #3[d]	2.8	1,750	4,818	1,925	40
surface impoundment Bethesda, Ohio[c]	0.5	2,000	986	610	62

(continued)

TABLE 13 (continued)

sample	organic-N before concentration (mg/L)	concentration factor	calculated organic-N value after concentration[a] (mg/L)	observed organic-N value after concentration (mg/L)	concentration efficiency[b] (%)
Concord River, (Bridge Street) Billerica, MA 7/4/78[c]	1.8	1,000	1,840	797	43
Middleton Pond, Danvers, MA (filtered to 0.1 μm) 7/11/78[c]	21.3	1,000	21,280	2,840	13
Middleton Pond, Danvers, MA (filtered to 0.1 μm) 7/11/78)[d]	21.7	1,000	21,681	2,448	11

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[a] Calculated organic-N value after concentration = organic-N value before concentration x concentration factor (assumes 100% recovery)

[b] Concentration efficiency = (observed organic-N value after concentration)/(calculated organic-N value after concentration)

[c] Filtered through XAD-8 and Tenax GC macroreticular resins

[d] Unfiltered

TABLE 14. RETENTION VALUES (ML) OF A MIXTURE CONTAINING 11 N-ORGANIC COMPOUNDS[a] ON ZIPAX SCX

elution position	.05 M KH_2PO_4 adjusted to pH 2.5 with H_3PO_4	.05 M K_2HPO_4 adjusted to pH 2.5 with H_3PO_4	.05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4	.05 M Na_2HPO_4 adjusted to pH 2.5 with H_3PO_4	.05 M NaH_2PO_4 pH 4.5	.01 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4
1	1.4	1.5	1.4	1.4	1.6	1.9
2	2.0	2.0	1.9	2.0	3.3	2.1
3	3.3	2.9	2.7	2.5	12.6	6.9
4	4.7	3.4	3.4	3.2	18.7	9.1
5	8.5	4.9	4.9	4.2	23.1	14.1
6	10.8	6.3	5.6	5.3	no other peaks observed after elution of additional 127 ml	20.0
7	17.5	12.8	6.7	6.7		26.0
8			8.6	11.6		31.8
9			12.0	15.9		41.7
10			20.1	no other peaks observed after elution of additional 100 ml		72.0
11			28.2			95.0
12						104.3

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[a] uracil, indole, alanine, tyrosine, purine, guanine, creatine, cytosine, adenine, creatinine, tryptophan; elution order of these compounds was not determined.

TABLE 15. RETENTION VALUES (ML) OF NITROGENOUS ORGANIC COMPOUNDS ON ZIPAX SCX (ONE METER COLUMN UNLESS OTHERWISE INDICATED)

Compound	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.01 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 50°C	H_2O adjusted to pH 2.5 with H_3PO_4	.005 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 10°C	.05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4	.05 M sodium perchlorate adjusted to pH 2.5 with perchloric acid	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 3 meter column	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 10% acetonitrile 3 meter column
1. d-ℓ serine	1.7				1.6					
2. uric acid	1.5			1.4				2.0		
3. barbituric acid	1.5							1.2		
4. succinimide	1.9				1.6			1.7	4.3	
5. ℓ-histidine	1.7				1.5					
6. ℓ-aspartic acid	1.6									
7. taurine	1.7			1.6						
8. uracil	1.5		1.0	1.4		1.3	1.5	1.3		
9. pyrrole	2.0							1.6		
10. 2,6,8, tri-chloropurine	1.9									
11. 5-chlorouracil	1.3									
12. thymine	1.5			1.6	1.6			1.4	4.6	4.4
13. ℓ-hydroxy-proline	1.9									
14. indole	2.2	2.0	1.4	1.4	2.1	2.1	2.0	1.7		
15. caffeine	2.4									
16. alanine	2.7	1.8	1.8	1.4	1.4 & 7.3	2.8	3.5	9.5		
17. glycine	2.1				1.6					
18. glycylglycine	2.4									
19. m-aminophenol	2.3 & 4.3									
20. pyrimidine	3.3			1.4		3.3		4.2		

(continued)

TABLE 15 (continued)

Compound	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.01 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 50°C	H_2O adjusted to pH 2.5 with H_3PO_4	.005 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 10°C	.05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4	.05 M sodium perchlorate adjusted to pH 2.5 with perchloric acid	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 3 meter column	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 10% acetonitrile 3 meter column
21. tyrosine	4.6		2.1			3.3	6.0	9.4		
22. purine	5.4		2.7			6.5	8.3	29.9		
23. guanine	6.0	5.8	3.9			7.7	10.5	38.8		
24. creatine	8.6		5.5			10.0		20.4		
25. cytosine	10.5	5.9				12.2	20.2	29.2	37.6	
26. adenine	12.5	7.0	7.6			17.2	22.8	31.9	45.8	
27. creatinine	15.0	7.8	8.6			18.9		26.5	53.1	
28. pyrrole	2.0								4.3	4.2
29. tryptophan	25.3		ND			ND	45.0		91.5	
30. urea	ND						ND			
31. humic acid	13.5									
32. phenyl-alanine							29.4			
33. ℓ-proline	20.3									
34. mixture: 8, 14, 16, 29, 21-27	all resolved		all resolved	only two initial peaks observed						
35. Marlboro W. concentrated effluent:	2.3						3.0 & 12.4			
spiked with:										
guanine	8.0						11.5			
cytosine	9.8						19.6			
creatinine	13.7						26.2			
tryptophan	17.9						42.0			

(continued)

TABLE 15 (continued)

Compound	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.01 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 50°C	H_2O adjusted to pH 2.5 with H_3PO_4	.005 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 temperature = 10°C	.05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4	.05 M sodium perchlorate adjusted to pH 2.5 with perchloric acid	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 3 meter column	.05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4 10% acetonitrile 3 meter column
36. SNPA[c] derivatized urine	1.4-10.5									
37. SNPA blank	1.7-13.0									

[a] Hyphenated values represent one large peak beginning and ending at the indicated values.

[b] ND = not detected

[c] SNPA = N-succinimidyl P-nitrophenylacetate (Regis Chemical Co.)

[d] Only 6 peaks observed in chromatogram of mixture of 11 compounds (8, 14, 16, 29, and 21-27) using 0.2 M $NH_4H_2PO_4$ (pH = 3.5) eluant.

TABLE 16. REPRODUCIBILITY OF RETENTION POSITIONS FOR SEVERAL NITROGENOUS COMPOUNDS ON ZIPAX SCX USING AN ELUANT OF .05 M $NH_4H_2PO_4$ ADJUSTED TO pH 2.5 with H_3PO_4

compound	replicate retention positions (ml)								mean (ml)	absolute standard deviation (ml)	percent standard deviation
	a	b	c	d	e	f	g	h			
uracil	1.3	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	0.04	3.08
indole	1.9	-	1.9	-	-	-	-	-	1.9	-	-
tyrosine	4.4	4.2	4.5	4.6	4.5	4.4	4.4	4.4	4.4	0.12	2.73
purine	5.3	5.1	5.4	5.6	5.5	5.3	5.4	5.4	5.4	0.15	2.78
guanine	5.9	5.6	6.0	6.2	6.0	5.9	5.9	5.8	5.9	0.17	2.88
cytosine	10.3	10.2	10.6	10.9	10.7	10.5	10.3	10.7	10.5	0.24	2.29
adenine	12.2	11.9	12.7	13.2	12.8	-	12.4	12.3	12.5	0.43	3.44
creatinine	15.0	15.0	15.1	15.7	15.1	15.3	14.8	14.7	15.1	0.31	2.05
tryptophan	29.2	27.3	29.7	31.3	29.7	-	29.2	28.2	29.2	1.26	4.32

compounds. Increased retention and subsequent resolution of some compounds in this group was achieved, however, on the Zorbax C8 column.

The reproducibilities of retention positions for several compounds are shown in Table 16. Although the absolute standard deviations were somewhat greater for slowly eluting compounds, the percentage standard deviations for these substances were not appreciably larger than for rapidly eluting materials. Absolute standard deviations ranged from 0.04 for a compound eluting at a mean position of 1.3 ml, to 1.26 for a compound eluting at a mean position of 29.2 ml. The corresponding percentage standard deviations for these two substances, however, were 3.08 and 4.32 respectively. Thus, while the absolute standard deviations varied by about 32-fold, the range in percentage standard deviation varied by a factor of about 1.4.

Injection of a mixture containing eleven compounds using a Zorbax CN column and .005 M paired ion exchange reagent mobile phase with or without 20% methanol (Table 17) produced only nine and six chromatographic peaks, respectively, suggesting that resolution was favored on the Zipax SCX column. The small number of peaks observed using the mobile phase without methanol was attributable to incomplete elution of the compounds by this weaker eluant. Elution and perhaps resolution of all the injected compounds might have been achieved by incremently increasing the methanol content of the mobile phase using a gradient making device. The large volume of mobile phase required to elute the most retentive compound from the column, illustrated the lack of rapid and clear resolution of constituent compounds using isocratic elution methods.

TABLE 17. RETENTION VALUES (ML) OF A MIXTURE CONTAINING 11 N-ORGANIC COMPOUNDS[a] ON ZORBAX CN USING PAIRED ION CHROMATOGRAPHY (PIC)

elution position	0.005 M PIC B7 1 heptane sulphonic acid; 20% methanol	.005 PIC B7 1 heptane sulphonic acid
1	2.7	2.9
2	3.1	3.2
3	4.0	4.1
4	5.0	6.2
5	6.8	8.3
6	16.3	26.6
7	32.0	no further peaks observed after elution with additional 242 ml mobile phase
8	157.0	
9	161.1	
	no further peaks observed after elution with additional 108 ml mobile phase	

[a] uracil, indole, alanine, tyrosine, purine, guanine, creatinine, cytosine, adenine, creatine, tryptophan; elution order of these compounds was not determined.

Tables 18 to 20 indicate the operating conditions for the resolution of 18 different nitrogenous compounds by isocratic elution technique. Resolution of all these compounds is not feasible using any one column and mobile phase but can be obtained from the chromatographic conditions shown in Table 20. Zorbax C8 proved to be very useful in separating a number of compounds which were unresolvable on the Zipax SCX column. Different retention characteristics were achieved by varying the pH of the buffered eluant. Increasing methanol content decreased the retentivity of compounds on the Zorbax C8 column. The strong retentivity observed for indole, using a small percentage of methanol in the eluant, suggested that gradient elution would facilitate resolution of mixtures of compounds within more reasonable periods of time.

Several chromatograms are included in Appendix B (Figures B-1 to B-4) to illustrate the varying adsorptivities of 11 compounds at three different wavelengths (220 nm, 233 nm, and 254 nm). The chromatograms also illustrate the phenomenon of peak spreading characteristic of isocratic elution techniques.

Maximum sensitivity was achieved on the U.V. monitor at an attenuation setting of 0.01 A.U. At this sensitivity setting, a full scale peak on the potentiometric chart recorder was equal to 1×10^{-2} absorbance units. The minimum detectable concentration of a trace compound was defined as the value which resulted in a peak height equal to 5% of a full scale deflection at the 0.01 A.U. sensitivity setting. Minimum detectable concentrations of nitrogenous compounds were then calculated according to the equation:

$$\begin{array}{c}\text{Minimum detectable concentration}\\ \text{at 0.01 A.U. attenuation setting}\\ \text{(moles/L)}\end{array} = \frac{(5 \times 10^{-4})}{E} \qquad (9)$$

where E is equal to the molear extinction value of a particular compound and the path length of the U.V. detector is equal to one cm. Minimum detectable concentrations for some nitrogenous organic compounds are shown in Table 21. Sensitivity settings of 2.0 or 1.0 A.U. were used when concentrated field and laboratory samples were analyzed, to minimize the number of response peaks going off the scale of the potentiometric recorder. Minimum detectable concentrations of nitrogenous materials monitored at these two settings were 2×10^2 and 1×10^2, respectively greater than those reported in Table 21.

Table A-37 (in Appendix A) illustrates the changing elution position of creatine with increasing concentration, attributable to column overloading. It is interesting to note that the creatine overloading did not effect the retention characteristics of other compounds introduced simultaneously indicating that saturation was occurring only at selected sites on the resin.

Later Studies: Gradient Elution--

A programmable gradient device was acquired later in this study to improve the resolution of concentrated field samples and to permit the separation of compounds having a wide range of polarities. Mixtures of reference compounds were not satisfactorily separated on either a Durrum DC 1-A resin using a pH 3, 0.2 M citrate buffer to 0.1 M citrate buffer (adjusted to pH 9.0 with NaOH) 20 minute, x^3 gradient, or an an Aminex A-27 column

TABLE 18. RETENTION VALUES (ML) OF NITROGENOUS COMPOUNDS ON ZORBAX C8 WITH CITRIC ACID BUFFER ELUANT

Compound	Mobile phase									
	100%.05 M pH 2.5 buffer	20% MeOH; 80% .05 M pH 2.5 buffer	50% MeOH; 50% .05 M pH 2.5 buffer	30% aceto-nitrile 70% 0.1 M pH 2.5 buffer	100% 0.1 M pH 2.5 buffer	75% MeOH; 25% .05 M pH 2.5 buffer	100% .05 M pH 2.0 buffer	50% MeOH; 50% .05 M pH 2.0 buffer	100% .05 M pH 3.9 buffer	50% MeOH; 50% 1.0 M pH 3.9 buffer
a. taurine	ND[b]					ND	ND	ND	ND	ND
b. ℓ-hydroxyproline	ND	ND				ND	ND	ND	ND	ND
c. indole	ND	47 & 52	13 & 14	34		4.9 & 5.1		12.9		
d. creatinine	ND		ND			2.7				
e. ℓ-aspartic acid	ND	ND				ND	ND	ND	ND	ND
f. ℓ-histidine	ND					ND	ND	ND	ND	ND
g. dℓ-serine	ND					ND	ND	ND	ND	ND
h. glycine	ND		ND			ND	ND	ND	ND	ND
i. barbituric acid	3.1	2.2								
j. guanine	3.8									
k. cytosine	3.3									
l. uracil	3.5									
m. uric acid	3.8								4.5	
n. succinimide	4.2	3.0							4.7	
o. tyrosine	5.8									
p. adenine	5.3									
q. 5-chlorouracil	6.4						5.8		7.3	
r. alanine									11.4	
s. purine	7.1								12.0	3.15
t. thymine	9.6	3.6			8.8		7.8	3.4	8.4	3.0
u. pyrrole	11.8	6.0		8.1	12.8		11.3	4.8	8.9	5.0
v. tryptophan	35.0									

(continued)

TABLE 18 (continued)

Compound	Mobile phase									
	100% .05 M pH 2.5 buffer	20% MeOH; 80% .05 M pH 2.5 buffer	50% MeOH; 50% .05 M pH 2.5 buffer	30% aceto-nitrile 70% 0.1 M pH 2.5 buffer	100% 0.1 M pH 2.5 buffer	75% MeOH; 25% .05 M pH 2.5 buffer	100% · .05 M pH 2.0 buffer	50% MeOH; 50% .05 M pH 2.0 buffer	100% .05 M pH 3.9 buffer	50% MeOH; 50% 1.0 M pH 3.9 buffer
Mixture i, m, n, q, t, & u	R[a]									
Raw Marlboro West pre-chlorinated secondary sewage effluent	3.0 & 5.3 NF[c] 150									
Marlboro West XAD filtered prechlorinated secondary sewage effluent	2.8 NF - 100									

[a] R - all components resolved
[b] ND - none detected
[c] NF - no further peaks observed after volume indicated

TABLE 19. RETENTION VALUES (ML) OF NITROGENOUS COMPOUNDS ON ZORBAX C8 WITH HIGHER pH BUFFER ELUANTS

compound	50% MeOH: 50% 0.2 M pH 6.9 PO_4 buffer	100% .05 M pH 6.9 PO_4 buffer	100% .01 M pH 6.9 PO_4 buffer	50% MeOH; 50% pH 8.9 borax buffer	100% pH 8.9 borax buffer
a. taurine	ND[b]			ND	ND
b. ℓ-hydroxyproline	ND			ND	ND
c. indole	15.9		265	14.5 & 15.2	
d. creatinine	2.8	4.4	4.9	2.65	4.1
e. ℓ-aspartic acid	ND			ND	ND
f. ℓ-histidine	ND			2.6	2.6
g. dℓ-serine	ND			2.7	2.7
h. glycine	ND			ND	ND
i. barbituric acid		3.4			2.4
j. guanine		5.6	2.6		5.3
k. cytosine		3.8	4.5		3.5
l. uracil		3.7	2.6		3.8
m. uric acid	ND	ND			
n. succinimide	2.8	5.1		3.3	4.1
o. tyrosine		4.5			4.4
p. adenine		15.4			14.5

(continued)

TABLE 19 (continued)

compound	50% MeOH; 50% 0.2 M pH 6.9 PO_4 buffer	100% .05 M pH 6.9 PO4 buffer	100% .01 M pH 6.9 PO_4 buffer	50% MeOH; 50% pH 8.9 borax buffer	100% pH 8.9 borax buffer
q. 5-chlorouracil	2.9	2.03			3.8
r. alanine		2.6	2.6		
s. purine		11.6			8.5
t. thymine	2.9	8.7		3.2	8.8
u. pyrrole	5.1	11.8		5.2	11.7
v. tryptophan	3.5	23.1			22.0
mixture d, i, j, q, s, u, p, v, & t		R[a]			
Marlboro West pre Cl_2 2° sewage effluent XAD filtered; concentrated x 200		One elution peak 1.5-8.0 ml			

[a] R = resolved

[b] ND = not detected

TABLE 20. COMPOUNDS RESOLVABLE[a] ON ZIPAX SCX OR ZORBAX C-8

compound	Zipax SCX .05 $NH_4H_2PO_4$	Zorbax C8 pH 2.5 citric acid buffer	Zorbax C8 pH 6.9 phosphate buffer	Zorbax C8 pH 8.9 borax buffer
taurine	1	ND[b]	ND	ND
ℓ-hydroxyproline	1	ND	ND	ND
indole	2	ND	11	10
creatinine	10	ND	3	4
ℓ-aspartic acid	1	ND	ND	ND
ℓ-histidine	1	ND	1	1
dℓ-serine	1	ND	1	1
glycine	1	ND	ND	ND
barbituric acid	1	1	2	1
guanine	6	1	5	5
cytosine	8	1	2	2
uracil	1	1	2	2
succinimide	1	2	5	4
tyrosine	4	3	4	4
adenine	9	3	9	9
5-chlorouracil	1	4	1	3
alanine	3	4	1	-
purine	5	5	7	6
thymine	1	6	6	6
pyrrole	1	7	8	7
tryptophan	11	8	10	8
creatine	7	-	-	-

[a] Numbers indicate elution order. Compounds having same number are unresolvable for conditions shown.

[b] ND = not detectable

TABLE 21. MINIMUM DETECTABLE CONCENTRATIONS OF SOME NITROGENOUS ORGANIC COMPOUNDS[a]

compound	wavelength	mean E[b]	standard deviation	minimum detectable concentration (MDC) moles/L	minimum detectable concentration (MDC) mg/L
cytosine	254	3.921×10^{3}	1.423×10^{3}	1.26×10^{-7}	9.57×10^{-3}
creatinine	254	1.074×10^{3}	319	4.66×10^{-7}	5.27×10^{-2}
indole	254	3.905×10^{3}	261	1.28×10^{-7}	1.50×10^{-2}
creatine	254	2.610×10^{1}	-	1.92×10^{-5}	2.51
creatine	233	1.454×10^{2}	-	3.44×10^{-6}	4.51×10^{-1}
creatine	210	2.365×10^{3}	431	2.11×10^{-7}	2.77×10^{-2}
uracil	254	7.779×10^{3}	275	6.43×10^{-8}	7.21×10^{-3}
uracil	233	2.215×10^{3}	26	2.27×10^{-7}	2.53×10^{-2}
alanine	254	2.70×10^{-1}	-	1.83×10^{-3}	165.0
tryptophan	278	6.559×10^{3}	384	7.62×10^{-8}	1.56×10^{-2}
adenine	256	1.388×10^{4}	279	3.60×10^{-8}	4.87×10^{-3}
succinimide	215	3.999×10^{2}	56	1.25×10^{-6}	1.24×10^{-1}
5-chlorouracil	272	8.183×10^{3}	395	6.11×10^{-8}	8.95×10^{-3}
pyrimidine	242	2.564×10^{3}	129	1.95×10^{-7}	1.56×10^{-2}
purine	260	7.603×10^{3}	193	6.58×10^{-8}	7.80×10^{-3}

[a] Peak height of 5 chart units at 0.01 A.U. attenuation setting

[b] Molar extinction values calculated from the mean values of standard solutions of nitrogenous compounds dissolved in ammonia-free water (pH unadjusted; see Table A-24).

using a 0.01 to 1.0 M ammonium-acetate-acetic acid buffer (pH = 4.4) gradient. Use of the Zorbax CN column with a pH 5.0, citrate buffer to 50% methanol, 20 minute gradient, resulted in the separation of eight compounds from a mixture of eleven materials. All eight resolved peaks, however, eluted rapidly and prior to the methanol phase of the gradient. Elution of a mixture of ten compounds (uracil, indole, pyrimidine, sarcosine, tyrosine, creatinine, cytosine, adenine, guanine, and tryptophan) on Zipax SCX (1 meter) using 0.01 M sodium perchlorate adjusted to pH 2.5 with perchloric acid eluant for 19 minutes followed by initiation of a 10 minute x^5 gradient to 1.0 M of the buffer, resulted in resolution of only seven of the compounds. Better resolution of this mixture was obtained using the Zipax SCX column with $NH_4H_2PO_4$ instead of the sodium perchlorate salt.

Successful separations of mixtures of reference compounds were obtained on either the Zorbax C-8 or the Zipax SCX column using the chromatographic conditions shown in Tables 22 and 23 and Figures 12 and 13. The Zorbax C-8 column exhibited greater selectivity for these compounds than did the Zipax SCX column. The C-8 column was therefore used most frequently in analyzing concentrated field samples.

TABLE 22. ELUTION POSITIONS[a] (MINUTES) OF REFERENCE NITROGENOUS COMPOUNDS ON ZIPAX SCX

Compound	0.01 - 1.0 M sodium perchlorate adjusted to pH 2.5 with perchloric acid; 10 minute, x^5 gradient	0.01 - 1.0 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4; 20 minute x^2 gradient
5-chlorouracil	1.1	-
indole	1.5	2.3
tyrosine	7.2	4.9
guanine	7.3	7.9
adenine	8.6	10.0
trptophan	25.9	13.1
uracil	-	1.8
alanine	-	4.0
purine	-	6.7
creatine	-	8.1
cytosine	-	10.2
creatinine	-	10.8

[a] Values represent mean retention times.

After several weeks, the Zorbax C-8 column (column 'a') began exhibiting peak aberations. This was later attributed to dissolution of the silica support in the analytical column, and the accumulation of colloidal or particulate material in the mobile phase or injected samples at the inlet of the chromatographic column. A new C-8 column (column 'b') was therefore obtained from the manufacturer. Although this replacement initially resulted in

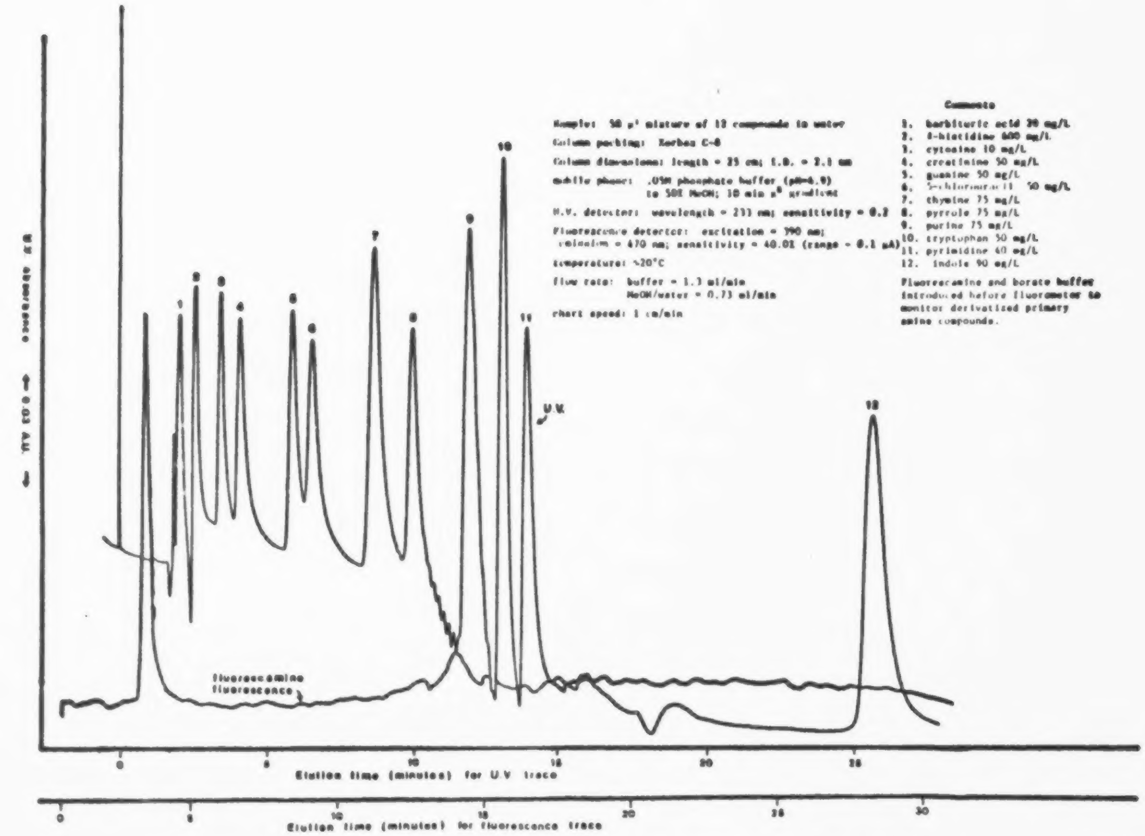

Figure 12. Chromatogram of a mixture of 12 nitrogenous compounds resolved on Zorbax C8 with a 0.05 M phosphate buffer (pH = 6.9) to 50% MeOH; 10 minute, X^5 gradient

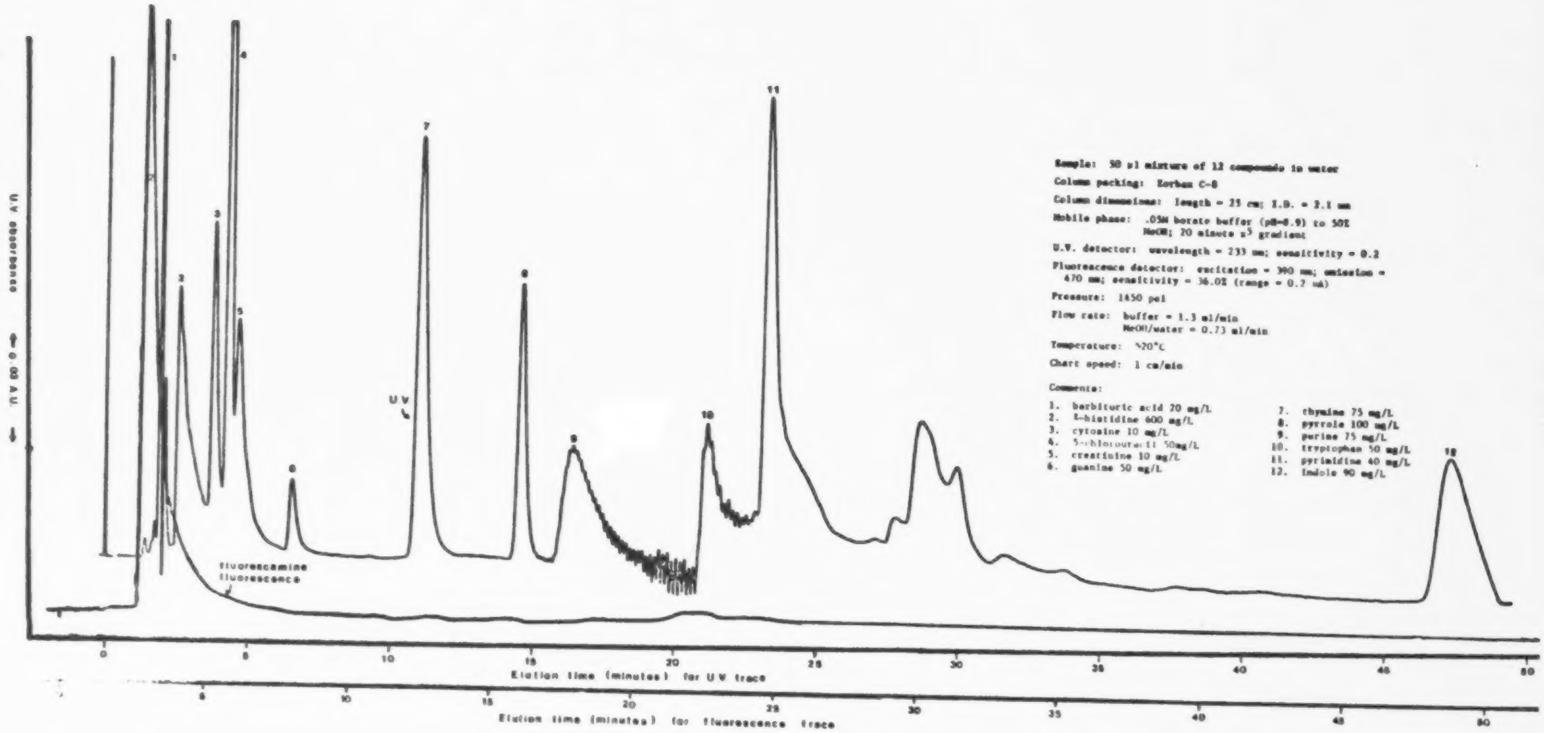

Figure 13. Chromatogram of a mixture of 12 nitrogenous compounds resolved on Zorbax C-8 with a 0.05 M borate buffer (pH=8.9) to 50% MeOH, 20 minute, X^5 gradient.

TABLE 23. ELUTION POSITIONS (MINUTES) OF REFERENCE NITROGENOUS COMPOUNDS ON ZORBAX C8 COLUMN "a", USING .05 M PHOSPHATE BUFFER (pH = 6.9) TO 50% MeOH, 10 MINUTE, x^5 GRADIENT

elution position[a]	compound	elution time (minutes)
1	urea	1.0
1	alanine	1.7
1	barbituric acid	1.8
1	creatine	2.1
1	uric acid	2.3
1	proline	2.5
2	ℓ-histidine	2.7
3	cytosine	3.5
3	uracil	3.5
3	tyrosine	3.5
4	succinimide	3.8
4	creatinine	3.9
5	guanine	5.2
6	5-chlorouracial	6.2
7	thymine	8.4
8	pyrrole	9.3
9	purine	11.7
10	tryptophan	12.9
10	adenine	13.1
11	pyrimidine	14.2
11	ℓ-hydroxyproline	16.0
12	indole	23.9

[a] Numbers indicate elution order. Compounds having same number are unresolvable for conditions shown. Values represent mean retention time.

separations similar to those attained on the original C-8 column (Table 24), loss in column retentivity was later observed after passage of about 20 to 50 ml of phosphate buffer through the column. Purine, for example, eluted more than twice as fast after the column had been in contact with about 30 ml of phosphate buffer in comparison to when the column was used immediately after it had been regenerated with methanol and reequilibrated with phosphate buffer. This occurrence was attributed to rapid bonding of trace contaminants in the phosphate buffer onto adsorption sites in the analytical column.

Loss of retentivity on the Zorbax C-8 column was observed to occur more slowly using a 0.05 M borate buffered eluant to 50% methanol gradient (Figure 13). Loss of retentivity was observed under these conditions only after several hundred milliliters of borate buffer had been eluted through the analytical column. This occurrence was again attributed to bonding of trace contaminants in the buffer onto adsorption sites of the chromatographic resin. Column retentivity was restored by regeneration with methanol.

TABLE 24. ELUTION POSITIONS[a] (MINUTES) OF REFERENCE NITROGENOUS COMPOUNDS ON ZORBAX C8, COLUMN "b", USING A .05 M PHOSPHATE BUFFER (pH = 6.9) TO 50% MeOH, 10 MINUTE, x^5 GRADIENT

elution position[b]	compound	elution time (minutes)
1	barbituric acid	2.0
1	ℓ-histidine	2.0
2	cytosine	3.2
3	creatinine	3.8
4	guanine	6.2
5	5-chlorouracil	7.9
6	thymine	9.8
7	pyrrole	12.6
8	purine	13.0
9	adenine	13.8
10	pyrimidine	15.5
11	indole	37.4

[a] Values represent mean retention times.

[b] Numbers indicate elution order. Compounds having same numbers are unresolvable for conditions shown.

With the exception of 5-chlorouracil, all of the reference compounds eluted in the same order using the borate buffered eluant (Table 25) as previously observed using the phosphate buffered mobile phase (Table 24). Thirteen compounds were resolved under these conditions. Most of the amino acids, however, were not strongly retained on the chromatographic column.

An unidentified chromatographic peak, eluting from about 27-30 minutes was observed in chromatograms of the mixture of reference compounds resolved on Zorbax C-8 using the borate buffered eluant (Figure 13). This was attributed to desorption by the methanol, of trace contaminants in the buffer which had been retained on the column during the first phase of the gradient.

A chromatogram of the ultraviolet and fluorescamine derivatized fluorescence traces obtained without injection of a sample is shown in Figure 14. The contaminant peak just discussed again appeared in the chromatogram during the methanol phase of the gradient. The fluorescence baseline remained quite flat during the buffer phase of the gradient but then increased by about 10% increasing methanol eluant composition. This was attributed to a corresponding pressure drop of about 56 to 50 p.s.i. in the post column lines. No baseline change was observed, however, in an underivatized fluorescence trace, obtained without injection of a sample, at a sensitivity setting two times greater than that used in monitoring the derivatized fluorescence baseline.

TABLE 25. ELUTION POSITIONS[a] (MINUTES) OF REFERENCE NITROGENOUS COMPOUNS ON ZORBAX C8, COLUMN "b", USING A .05 M BORATE BUFFER (pH 8.9) TO 50% MeOH, 20 MINUTE, x^5 GRADIENT

elution position[b]	compound	elution time (minutes)
1	aspartic acid	1.5
1	glycine	1.8
1	glutamic acid	1.7
1	serine	1.8
1	barbituric acid	1.9
1	thioruea	2.0
1	glycylglycine	2.0
2	proline	2.5
2	lysine	2.6
2	valine	2.7
2	ℓ-histidine	2.8
3	succinimide	3.4
3	tyrosine	3.7
3	cytosine	3.7
4	leucine	4.3
4	uracil	4.1
4	5-chlorouracil	4.3
5	creatinine	4.6
6	guanine	6.3
7	thymine	10.5
8	pyrrole	14.4
8	phenylalanine	14.2
9	purine	15.5
10	adenine	21.1
10	tryptophan	21.0
11	pyrimidine	23.0
12	indole	46.3
13	skatole	67.5

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[a] Values represent mean retention times.

[b] Numbers indicate elution order. Compounds having same numbers are unresolvable for conditions shown.

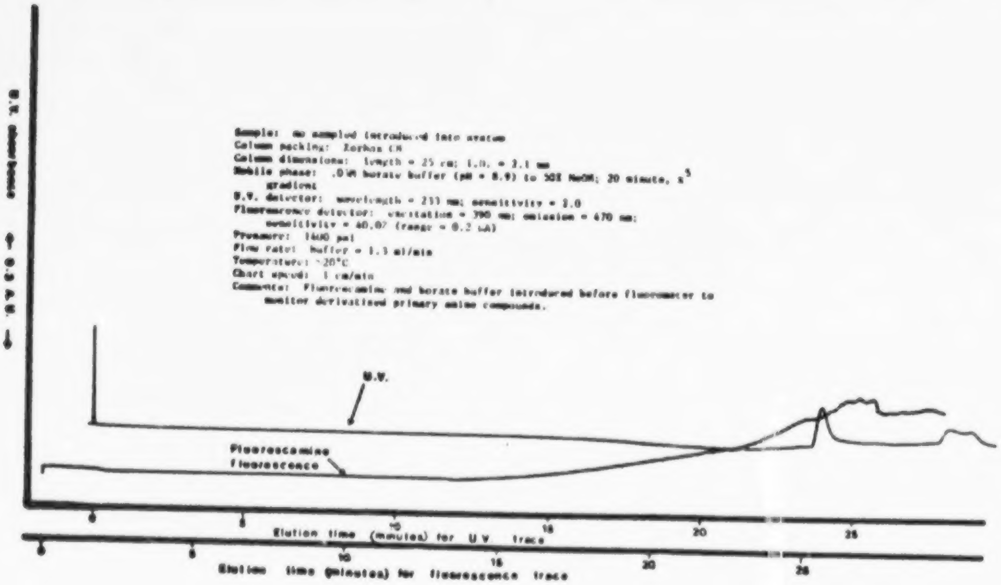


Figure 14. Chromatogram of U.V. and fluorescamine-derivatized fluorescence traces obtained without injection of a sample.

The Zorbax C-8 'b' column began exhibiting peak aberration after being used for only several days. The analytical life of this column, using the borate buffered eluant, was shorter than that of the Zorbax C-8 'a' column, using phosphate buffered eluant. This was attributed to the more rapid dissolution of the silica support in the column using the more alkaline mobile phase.

Another replacement column (column 'c') was therefore obtained from the manufacturer and used with a guard column. The guard column prevented fouling of the analytical column by sample or mobile phase contaminants. In addition, the silica-containing guard column presaturated the mobile phase with dissolved silica, thereby decreasing the rate of silica dissolution in the analytical column. The guard column consisted of a 4.6 i.d. by 5 cm stainless steel tube packed with permaphase ODS (DuPont Co.) and fitted with a 20 μm inlet frit and 2 μm outlet frit. Elution positions for some reference nitrogenous compounds on the Zorbax C-8 'c' column using a 0.05 M borate buffer (pH = 8.9) to 50% methanol, twenty minute, x^5 gradient and the 5 cm permaphase guard column are shown in Table 26. With the exception of purine all of the reference compounds exhibited the same retention times using the borate buffer with the guard column (Table 26) as previously observed without the guard column (Table 25). Purine eluted slightly more rapidly using the guard column and the new Zorbax C-8 column, and was therefore not resolvable from pyrrole. The guard column did not appear to decrease the overall separation efficiency to any observable extent.

TABLE 26. ELUTION POSITIONS[a] (MINUTES) OF REFERENCE NITROGENOUS COMPOUNDS ON ZORBAX C8 COLUMN "c", USING A .05 M BORATE BUFFER (pH 8.9) TO 50% MeOH, 20 MINUTE x^5 GRADIENT (USING A 5 cm PERMAPHASE GUARD COLUMN)

elution position[b]	column	elution time (minutes)
1	barbituric acid	2.3
2	ℓ-histidine	3.3
3	cytosine	3.9
4	5-chlorouracil	4.2
4	uracil	4.3
5	creatinine	4.9
6	guanine	6.5
7	thymine	10.7
8	pyrrole	14.0
8	purine	14.0
9	adenine	21.6
10	tryptophan	22.5
11	pyrimidine	23.8

[a] Values represent mean retention times.

[b] Numbers indicate elution order. Compounds having same numbers are unresolvable for conditions shown.

Ultraviolet spectra of reference nitrogenous compounds were recorded after correction for the background spectrum of 50% methanol in water, the flow cell and photomultiplier, using the memory module. Figures 15 and 16 show the uncorrected and corrected spectra, respectively, of 50% methanol in water. Figures 17 to 30 and 31 to 56 show the corrected spectra of reference nitrogenous compounds eluted through Zorbax C-8 using a 0.05 M phosphate buffer (pH = 6.9) to 50% methanol, 10 minute, x^5 gradient and a 0.05 M sodium borate buffer (pH = 8.9 with H_3PO_4) to 50% methanol, 20 minute, x^5 gradient, respectively. Neither of the two buffers had significant absorbance at wavelengths greater than about 225 nm for the signal attenuation used in the spectral scans (Figures 57 and 58). End absorbance at wavelengths less than 225 nm for compounds eluting in the buffer phase of the gradient program was partially attributable to absorbance by the buffer. Most of the reference nitrogenous compounds exhibited the same U.V. spectra in the phosphate buffer as in the borate buffer. Shifts in the positions of maximum and minimum absorbancies attributable to the different buffers were noted, however, for purine, succinimide, tyrosine, and 5-chlorouracil. Spectral data for some of the reference compounds are summarized in Table 27.

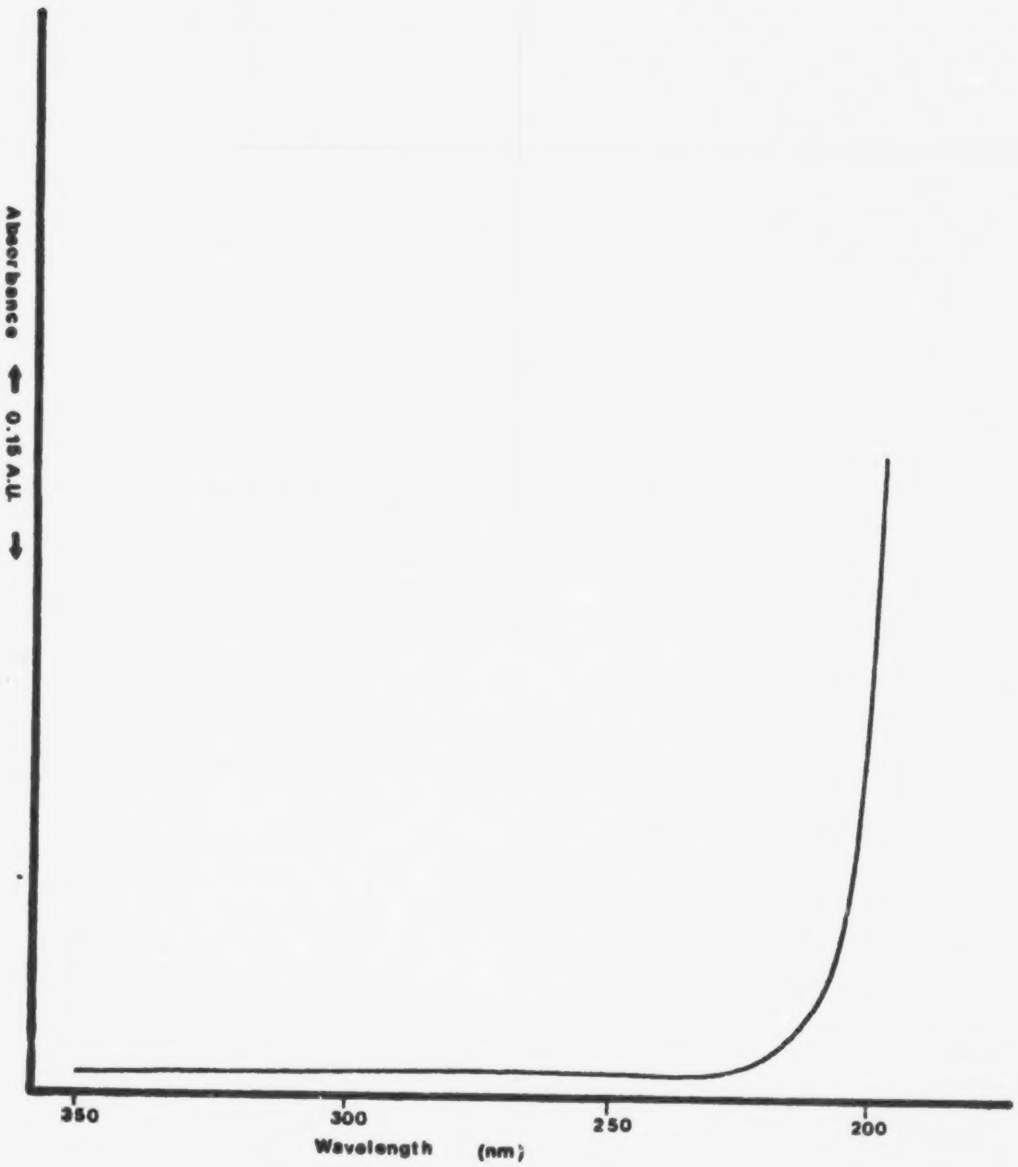


Figure 15. 50% methanol uncorrected signal.

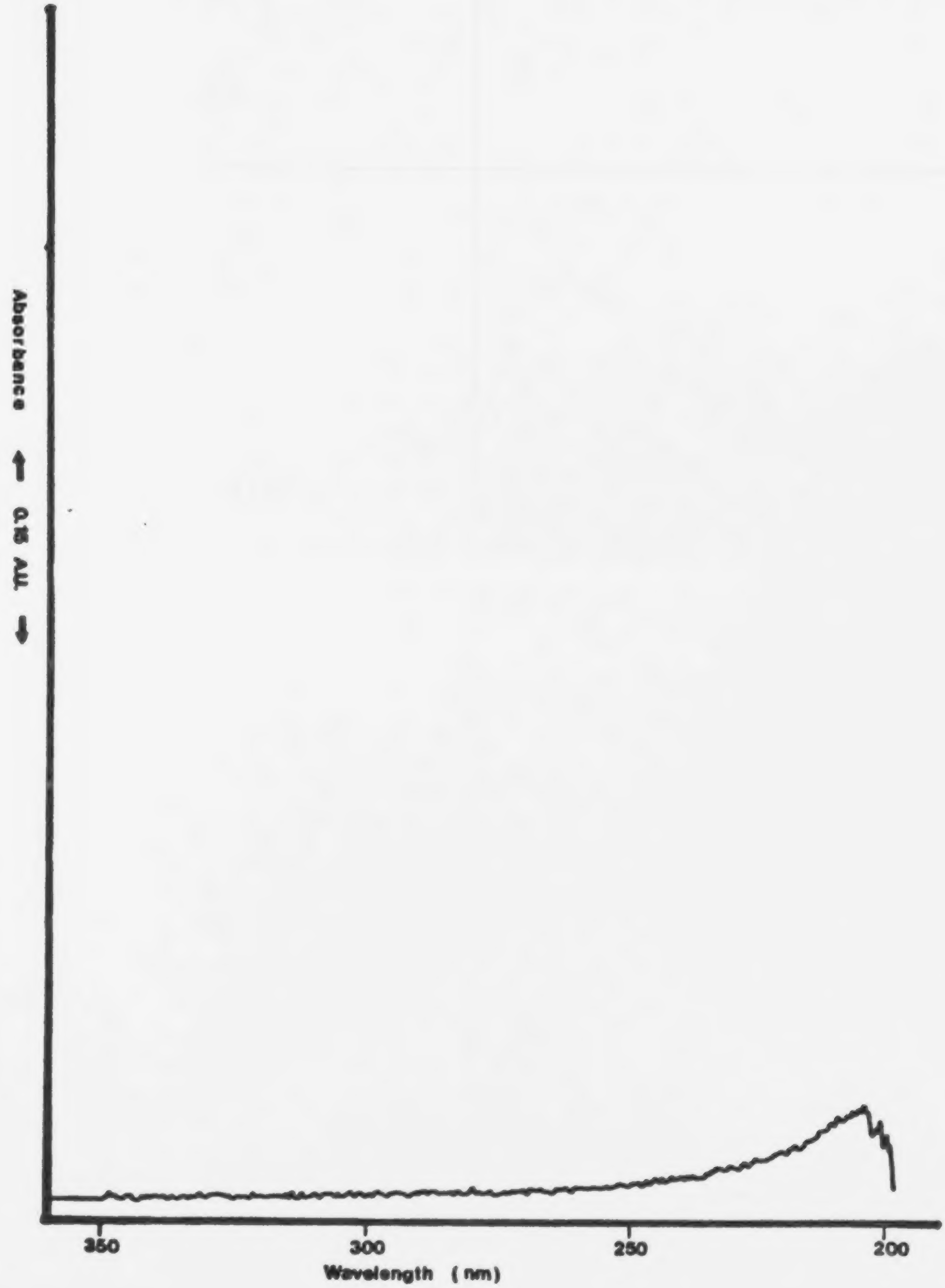


Figure 16. 50% methanol corrected signal.

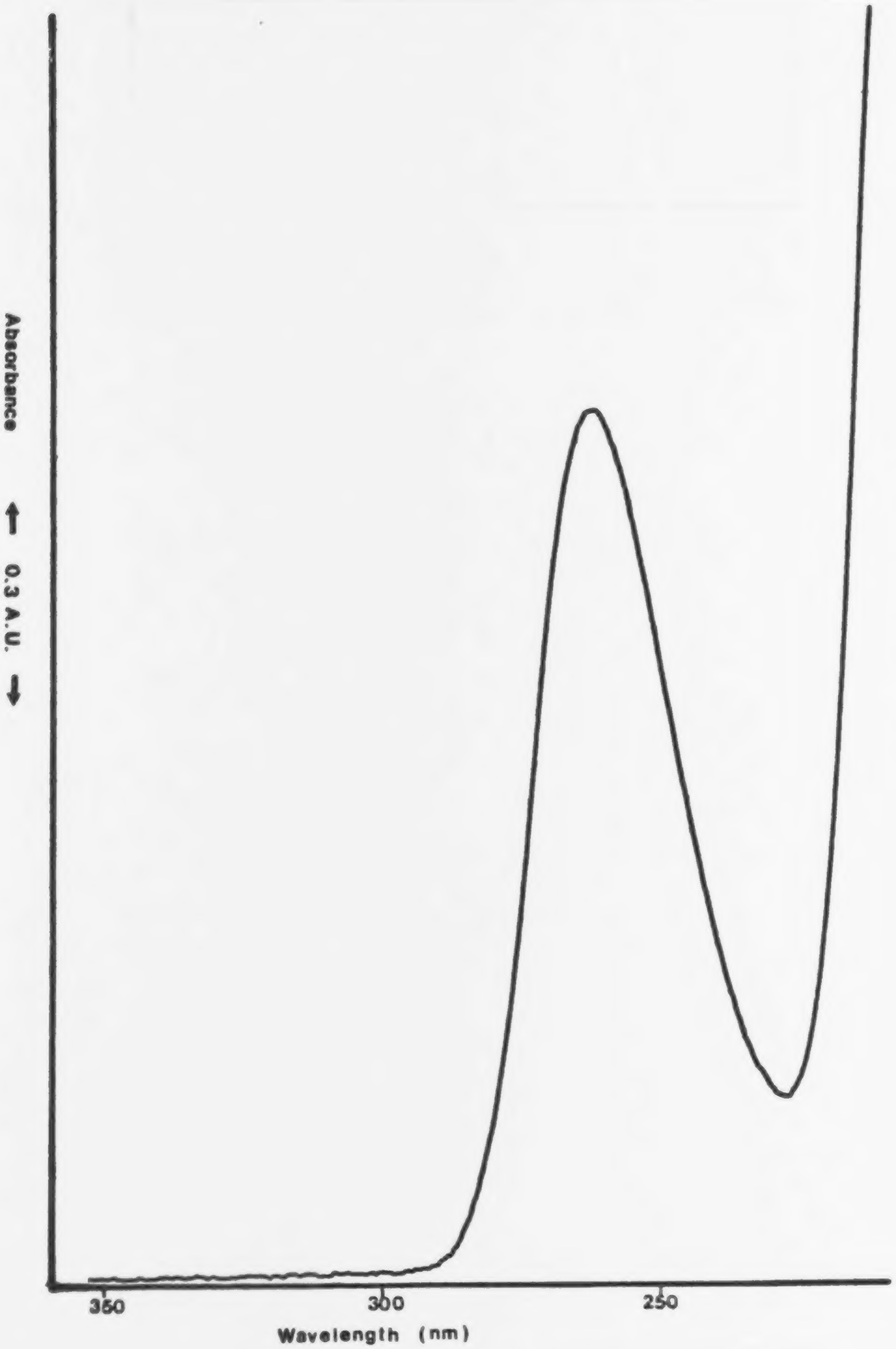


Figure 17. Adenine (in 0.05 M phosphate buffer).

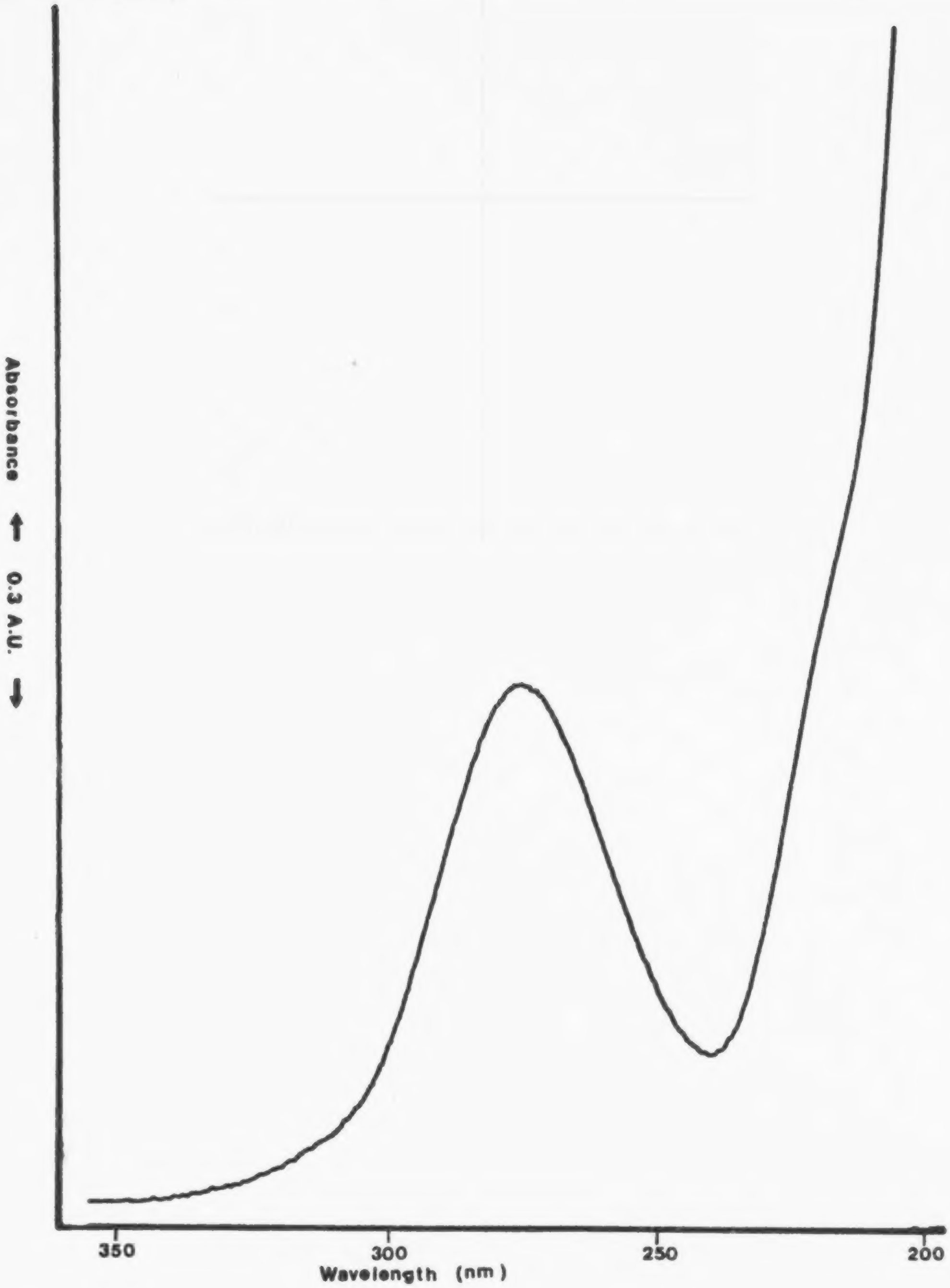


Figure 18. 5-chlorouracil (in phosphate buffer).

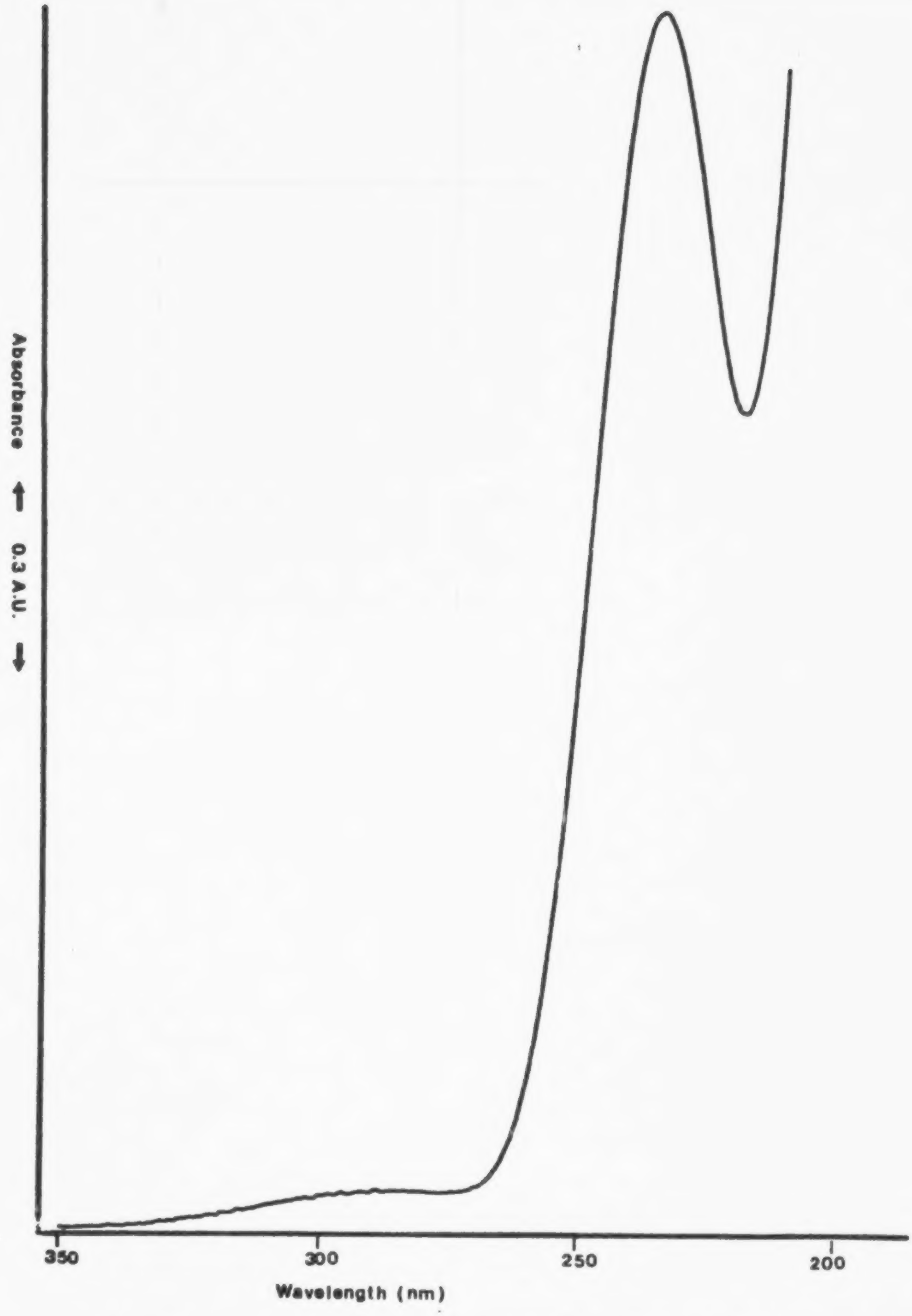


Figure 19. Creatinine (in 0.05 M phosphate buffer).

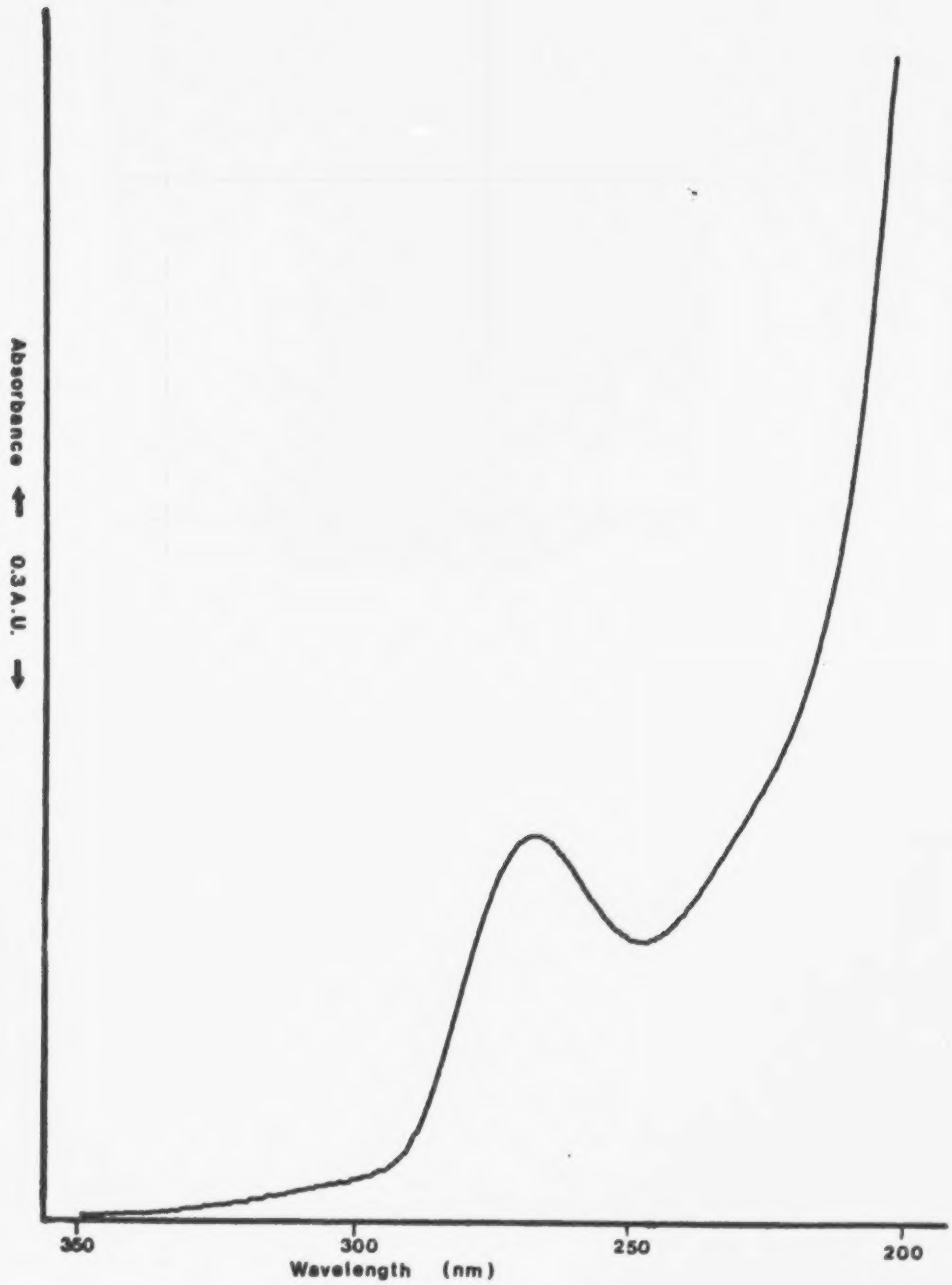


Figure 20. Cytosine (in 0.05 M phosphate buffer).

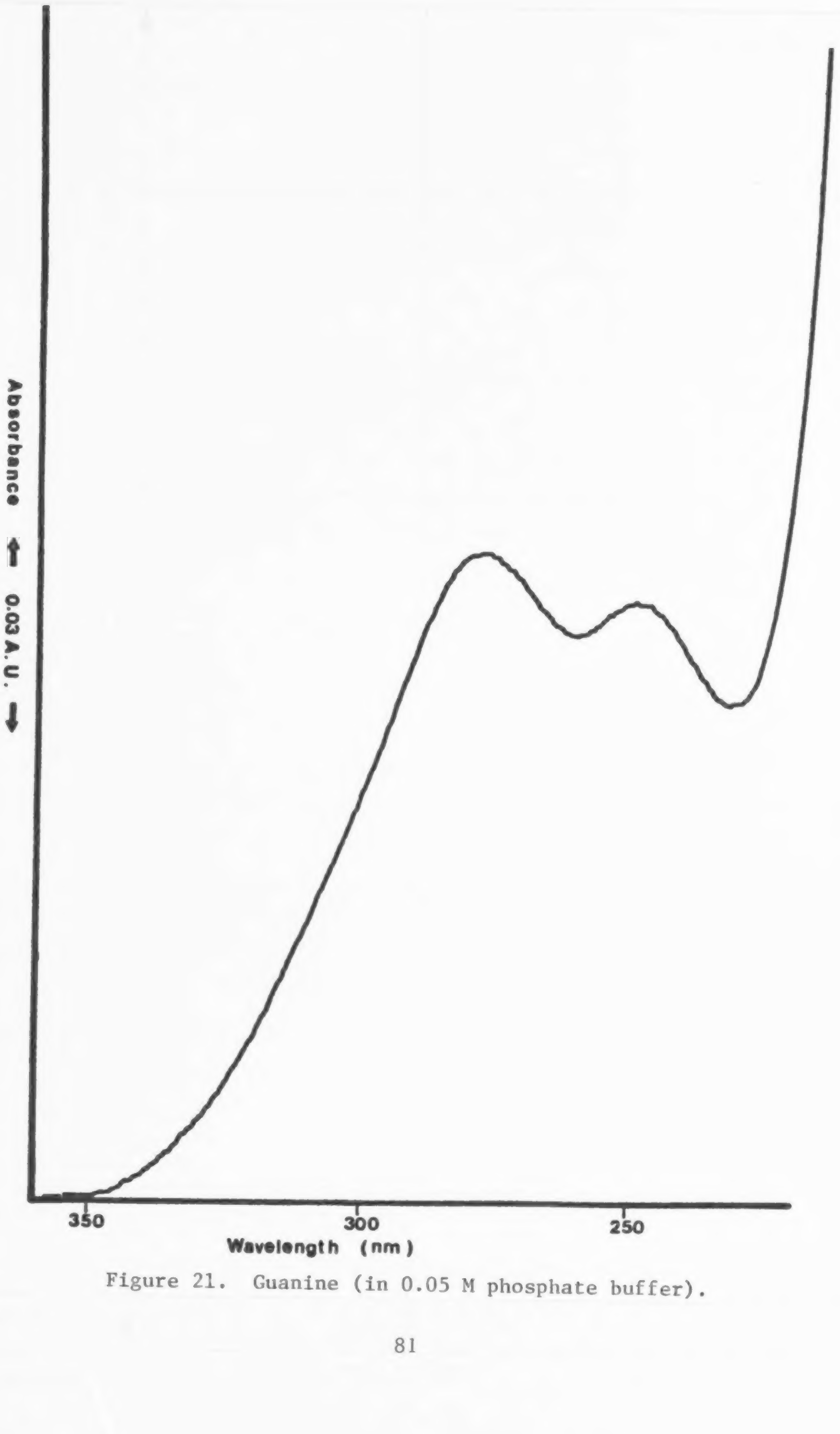


Figure 21. Guanine (in 0.05 M phosphate buffer).

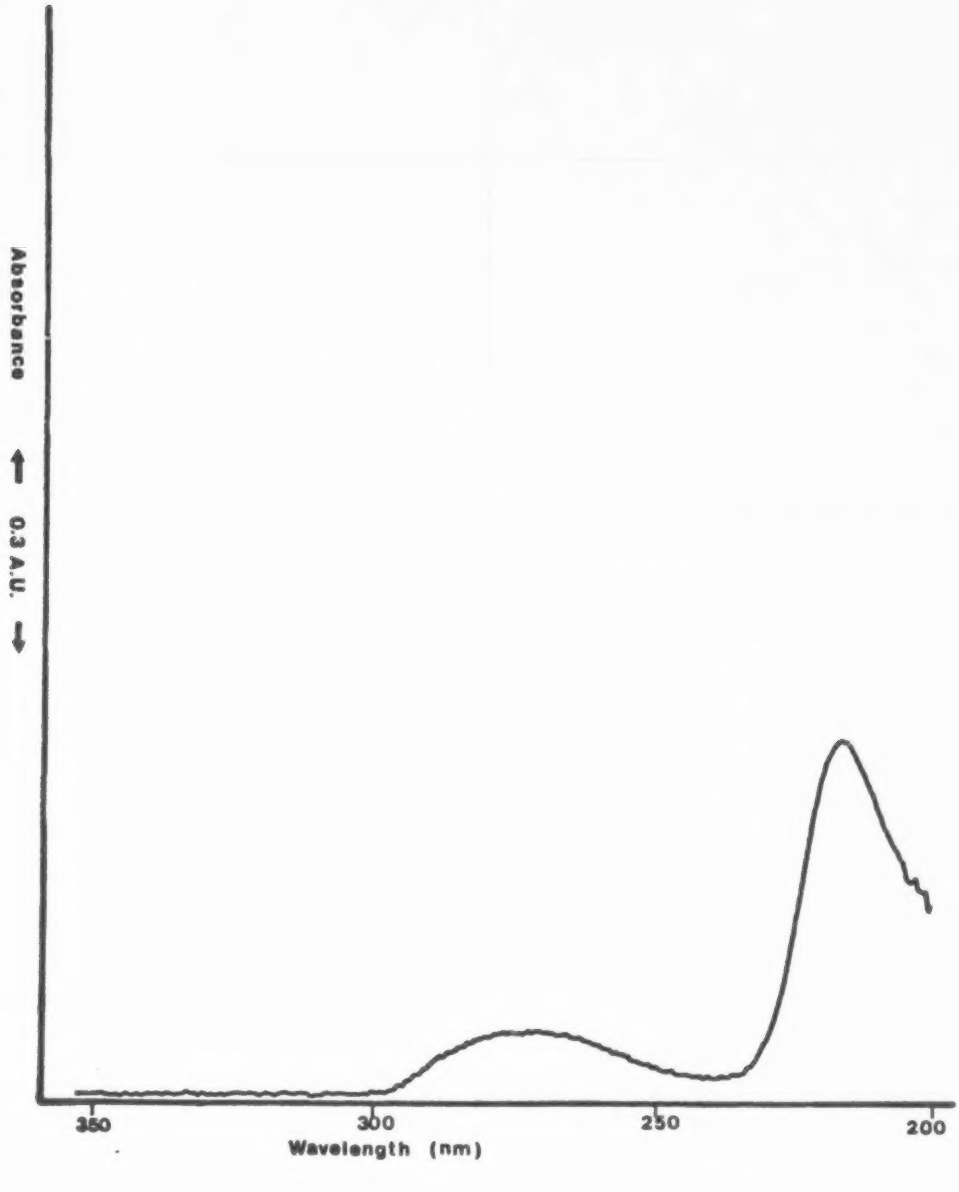


Figure 22. Indole (in 0.05 M phosphate buffer).

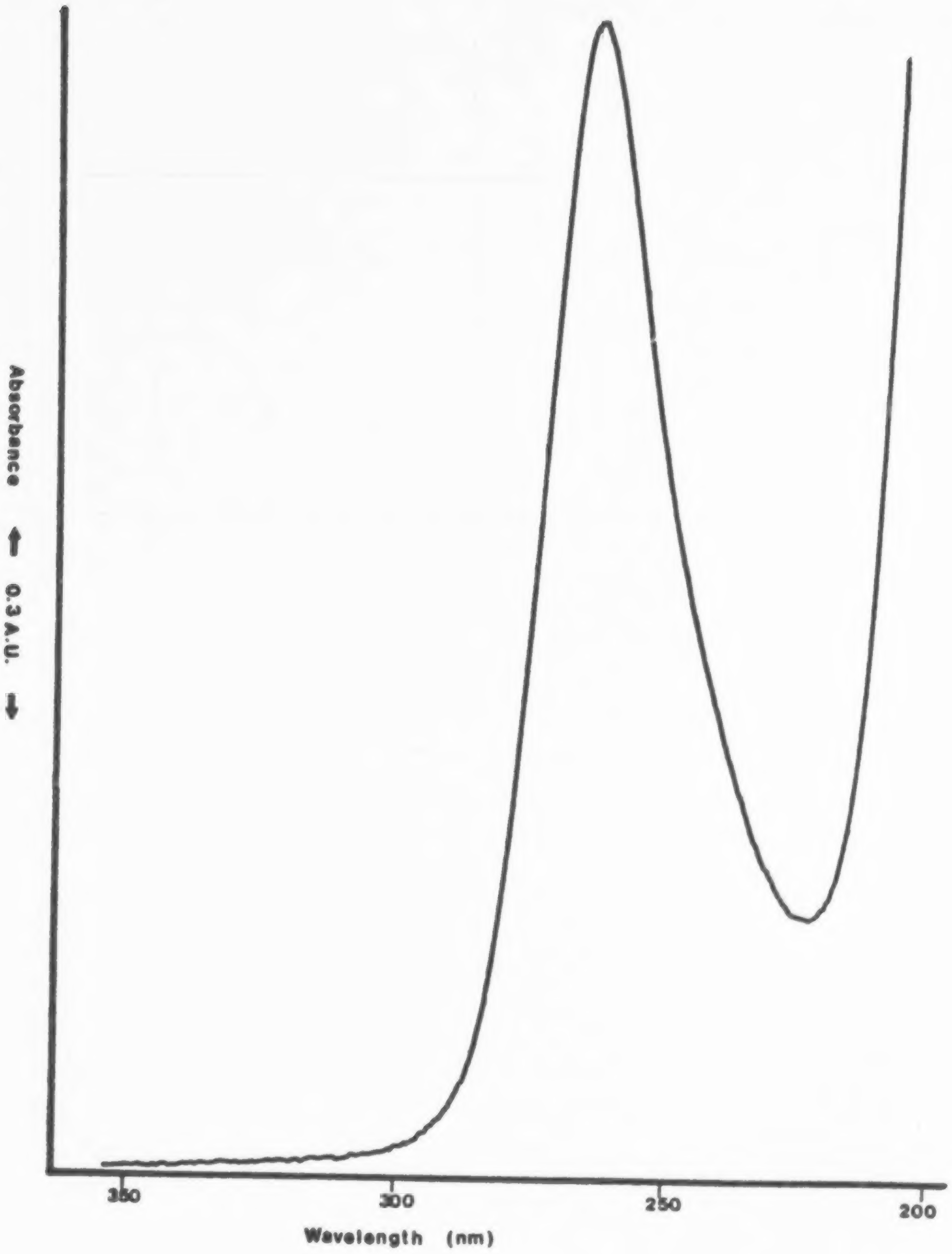


Figure 23. Purine (in 0.05 M phosphate buffer).

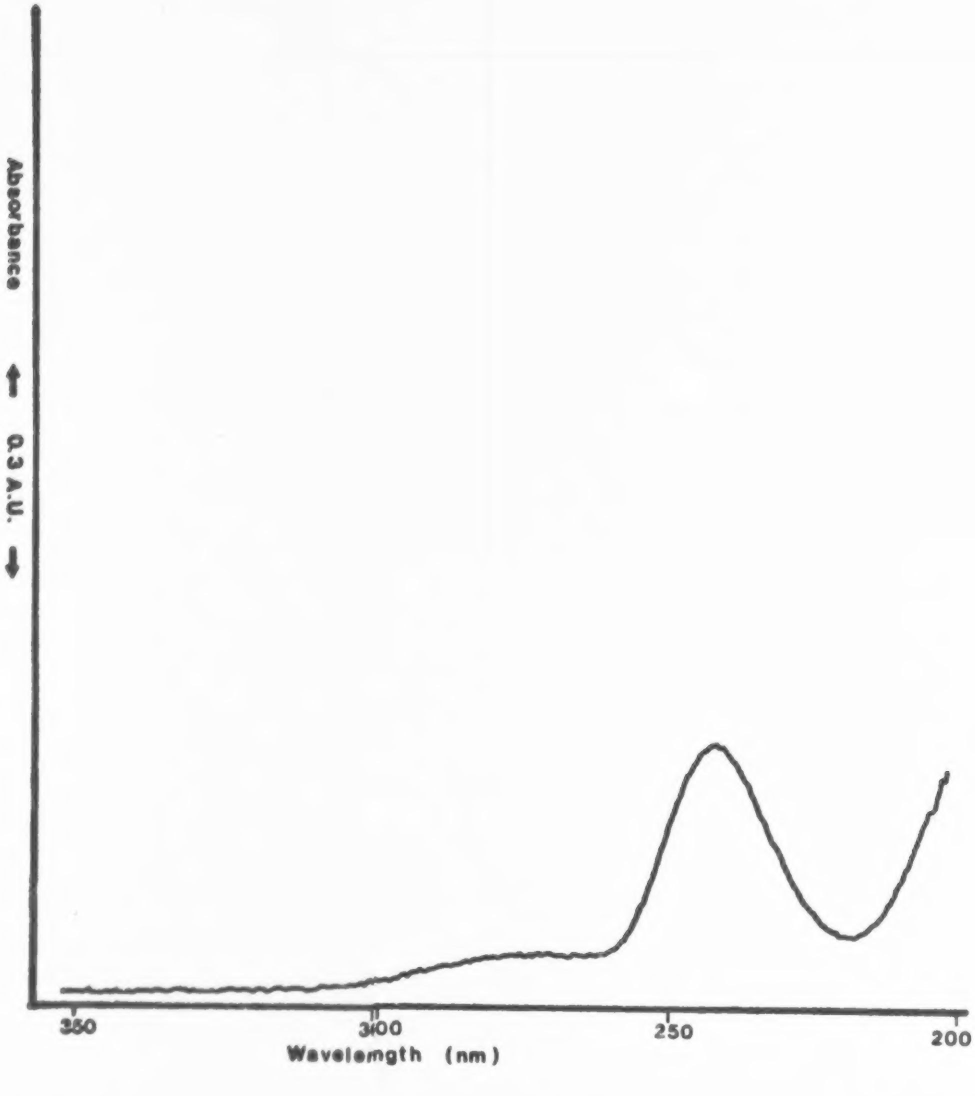


Figure 24. Pyrimidine (in 0.05 M phosphate buffer).

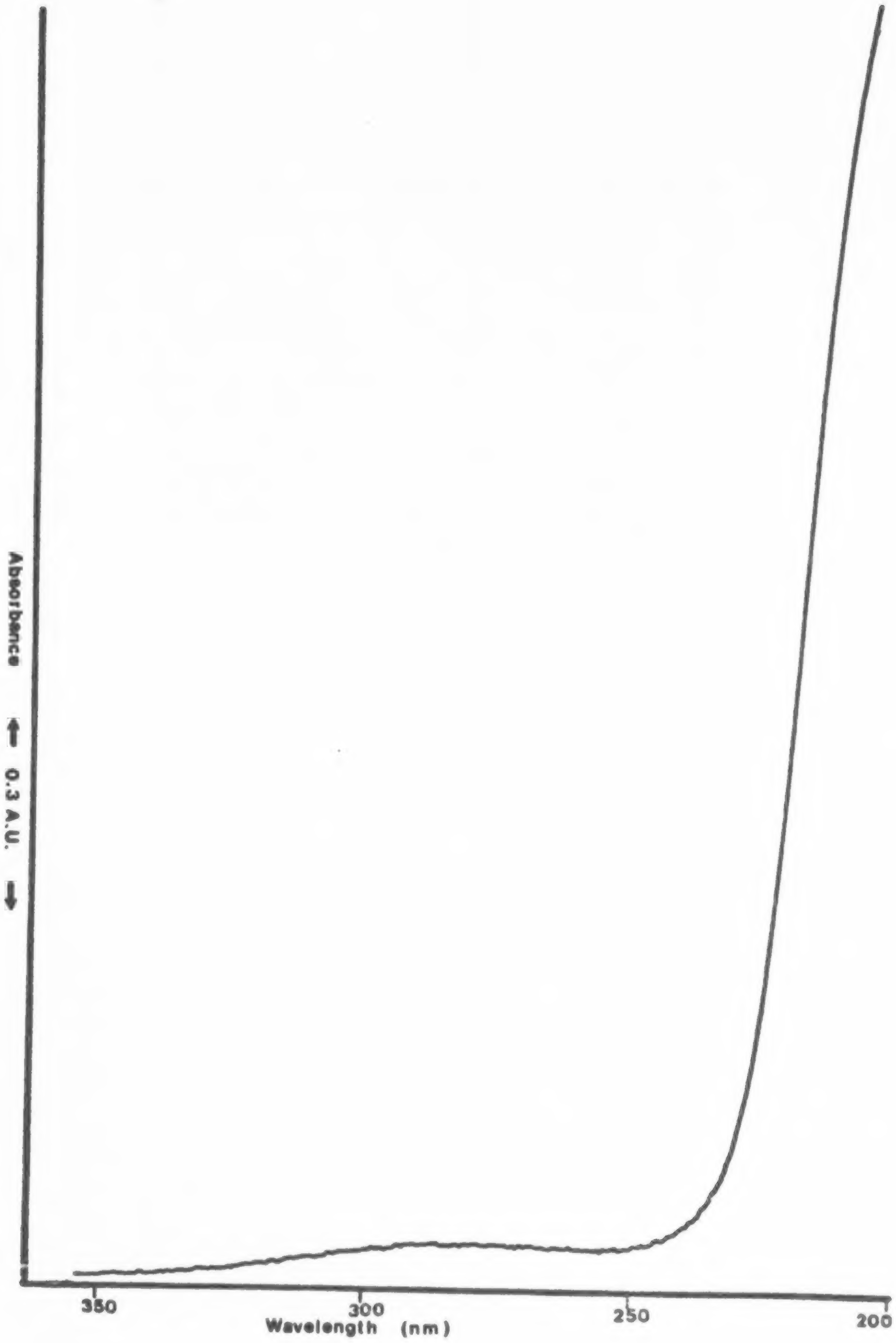


Figure 25. Pyrrole (in 0.05 M phosphate buffer).

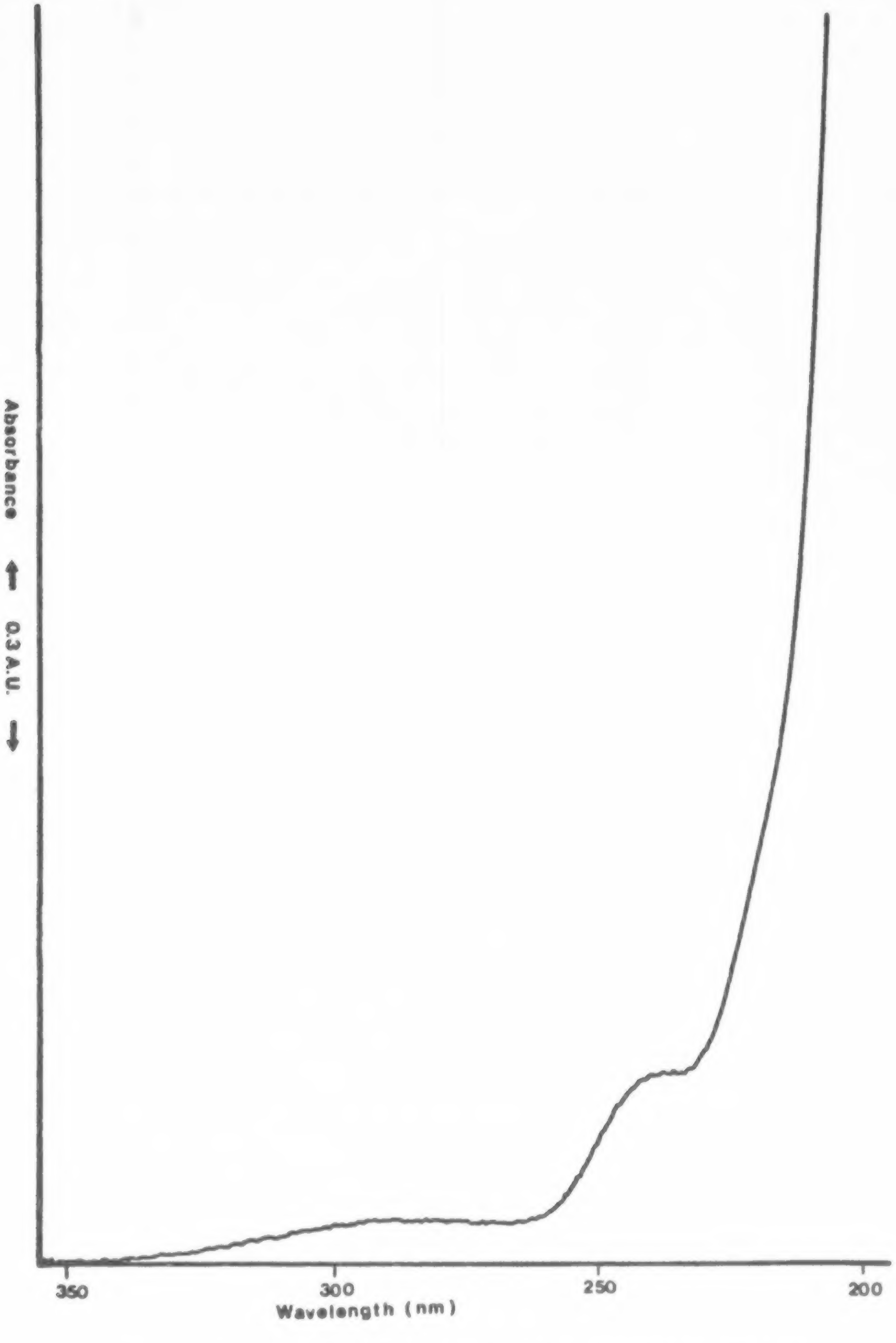


Figure 26. Succinimide (in 0.05 M phosphate buffer).

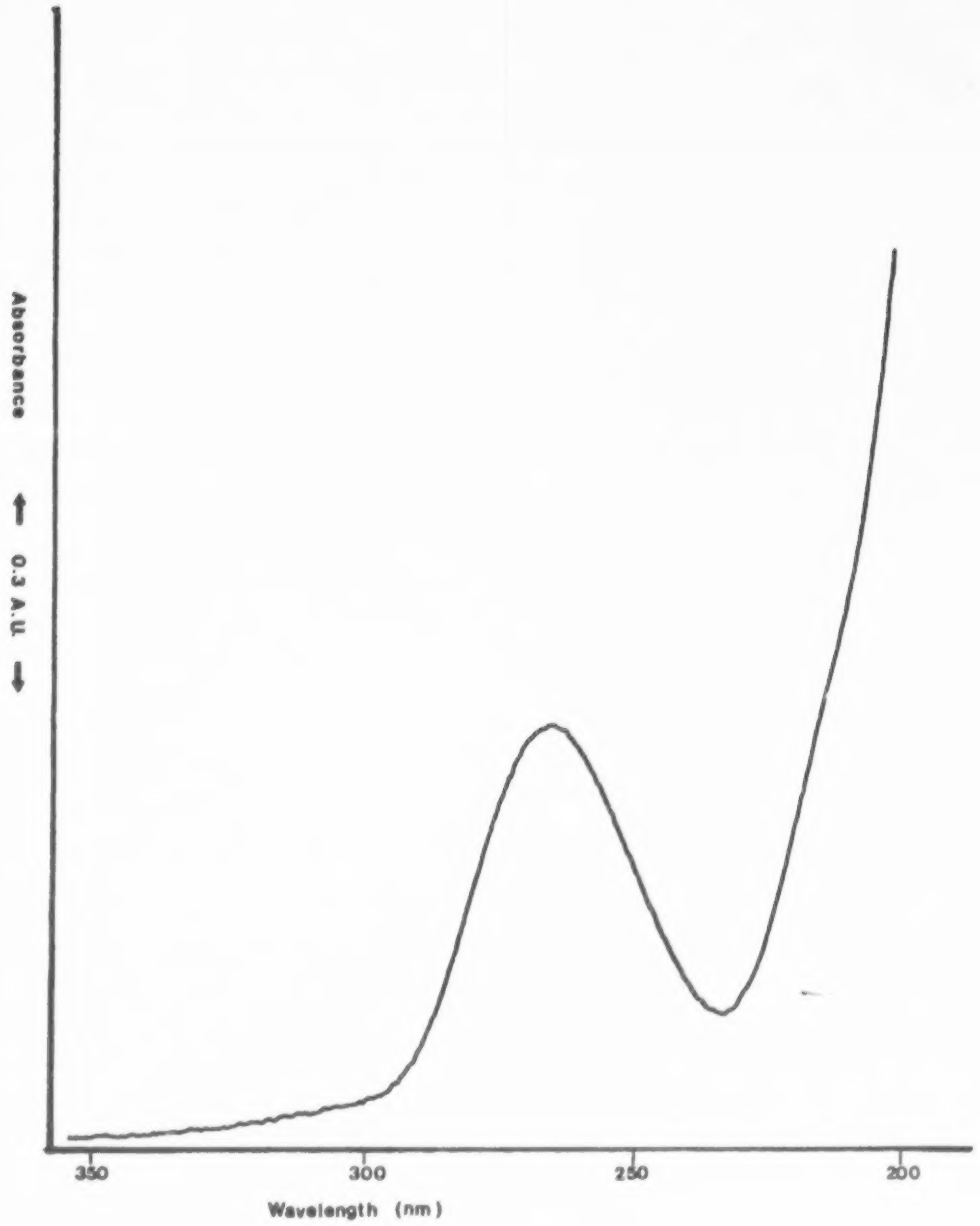


Figure 27. Thymine (in 0.05 M phosphate buffer).

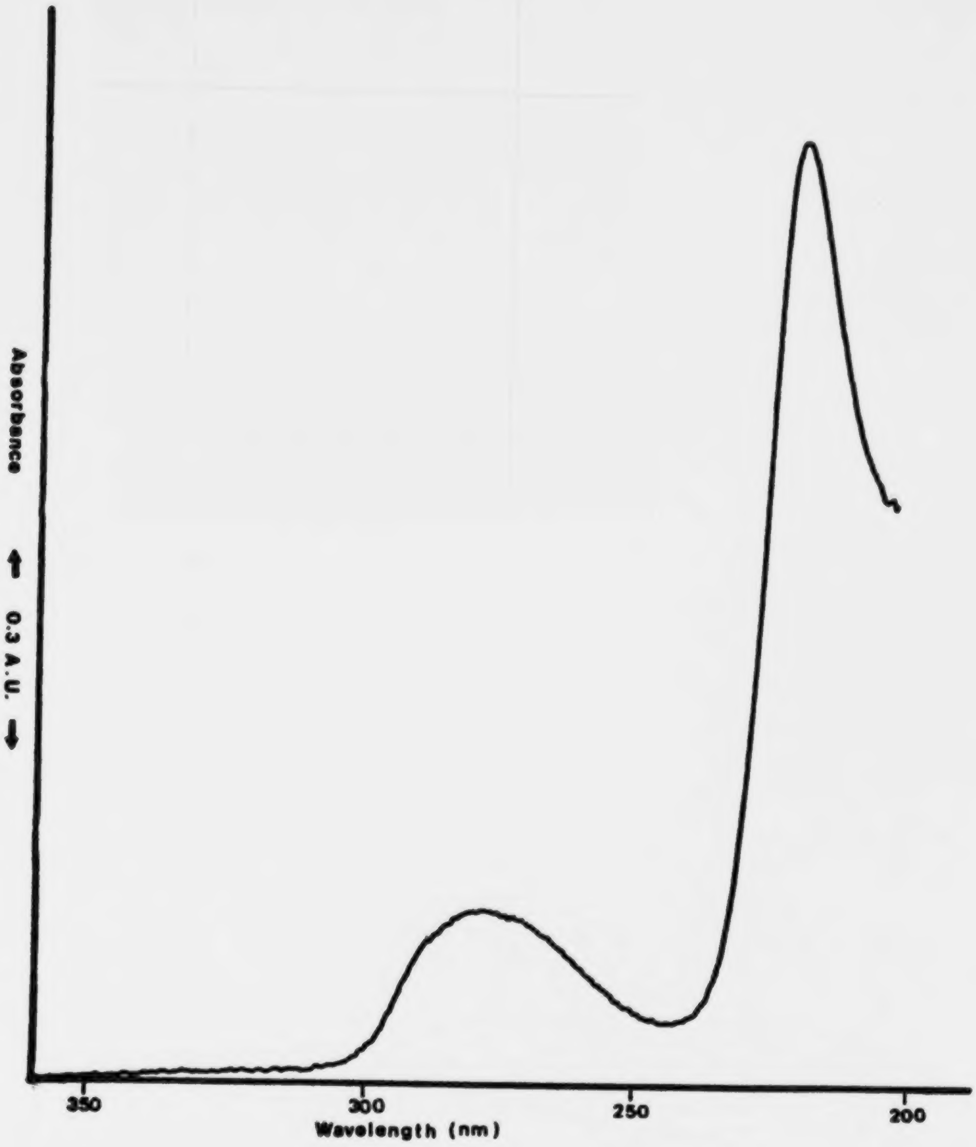


Figure 28. Tryptophan (in 0.05 M phosphate buffer).

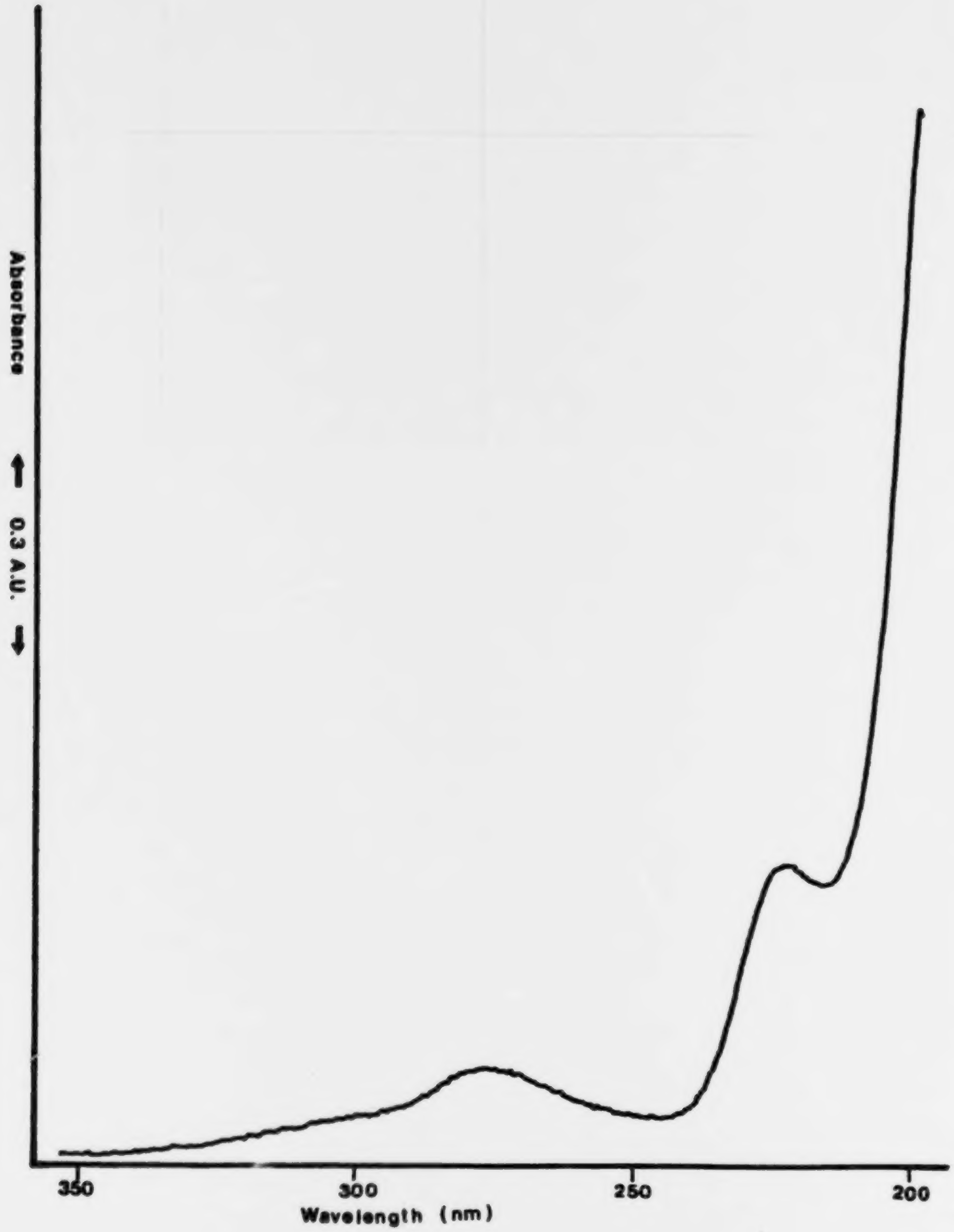


Figure 29. Tyrosine (in 0.05 M phosphate buffer).

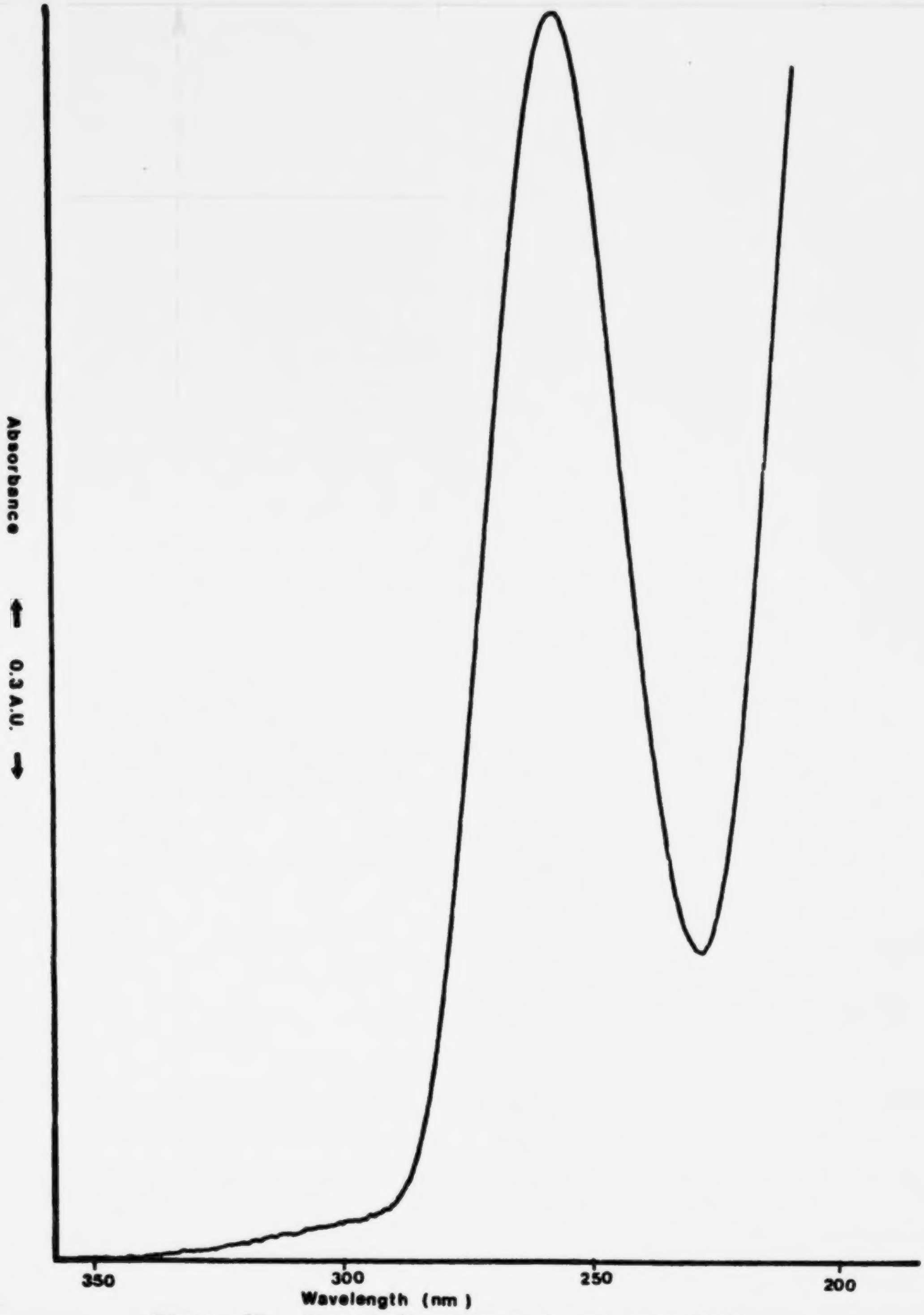


Figure 30. Uracil (in 0.05 M phosphate buffer).

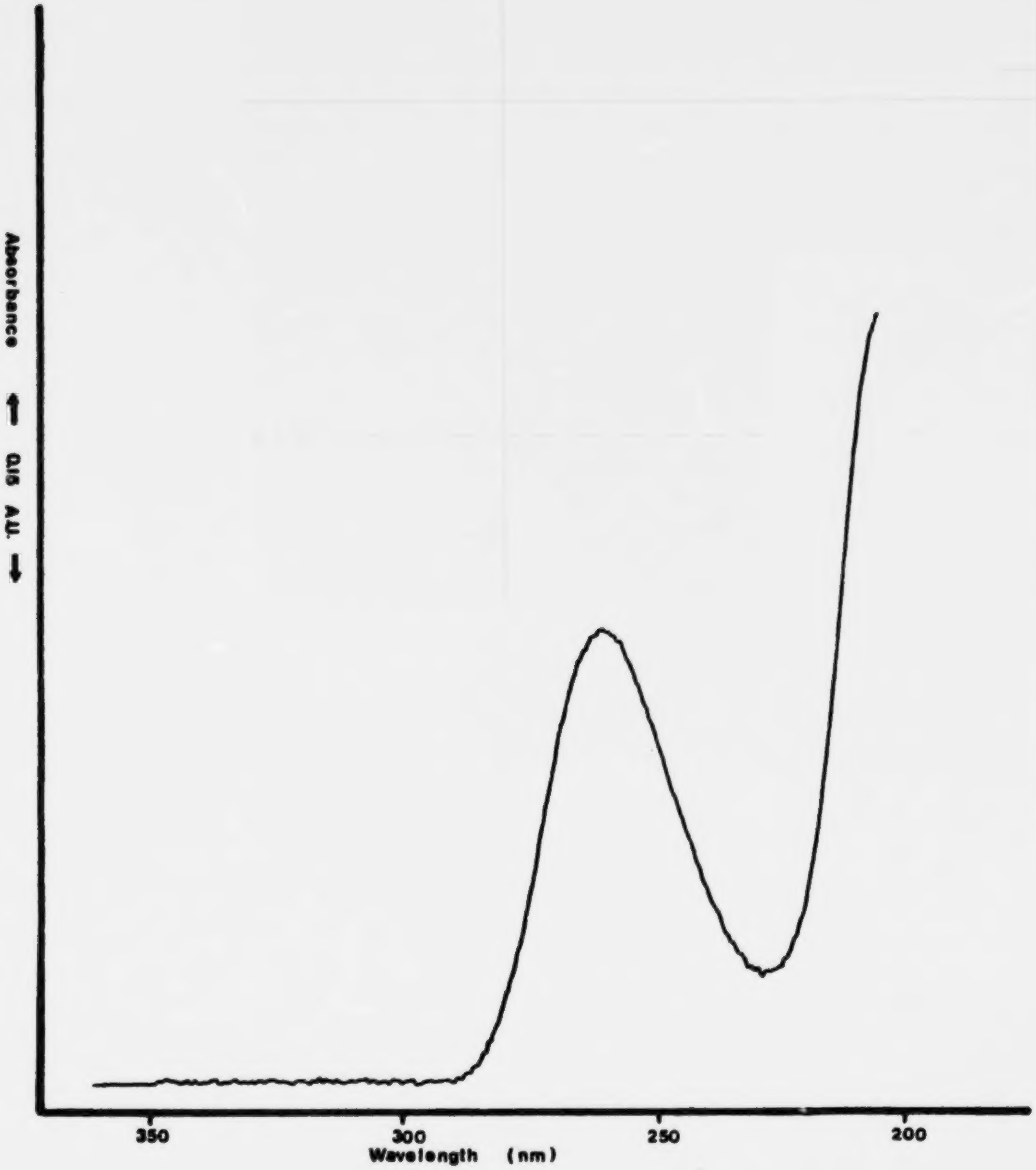


Figure 31. Adenine (in 0.05 M borate buffer).

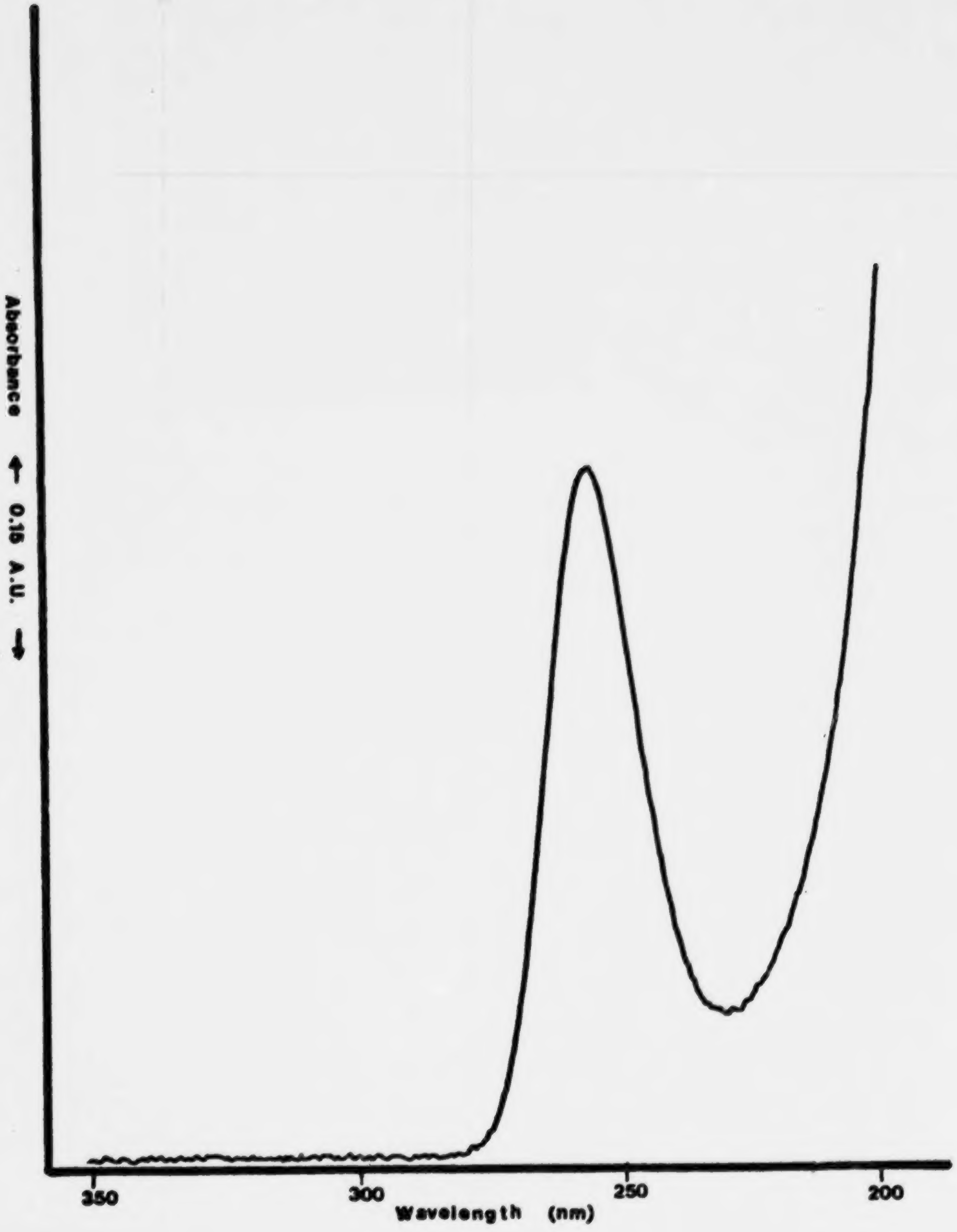


Figure 32. Barbituric Acid (in 0.05 M borate buffer).

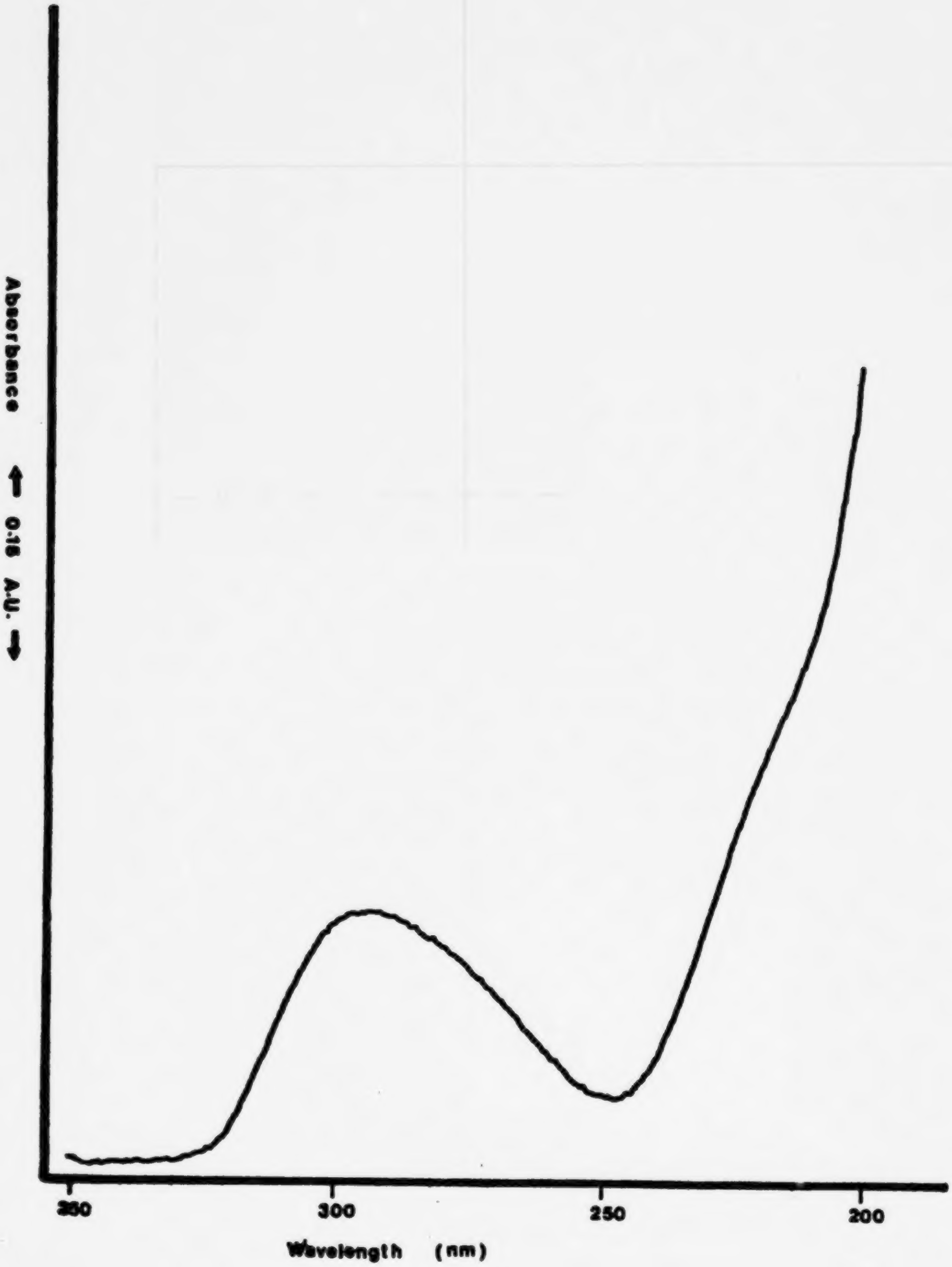


Figure 33. 5-chlorouracil (in borate buffer).

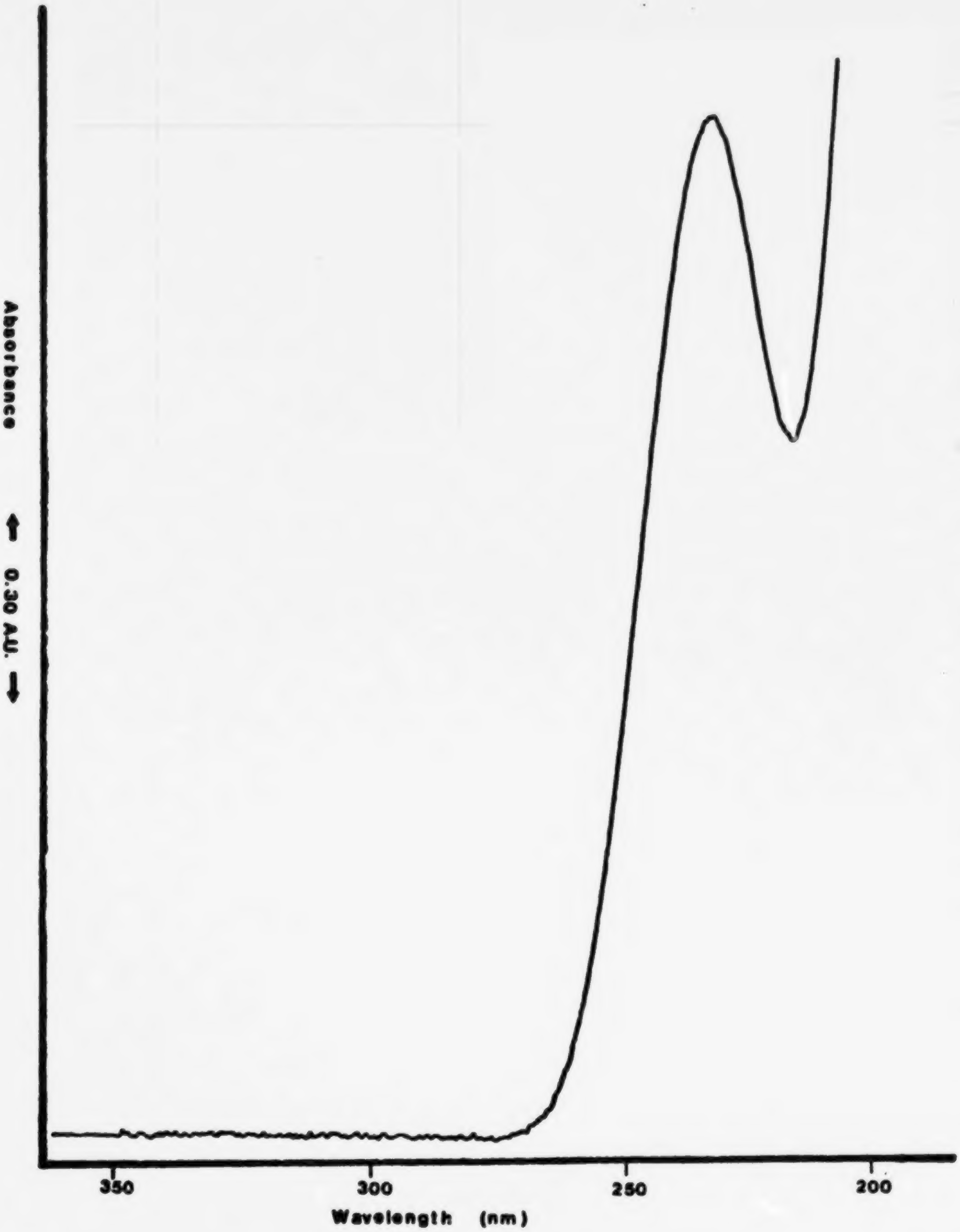


Figure 34. Creatinine (in 0.05 M borate buffer).

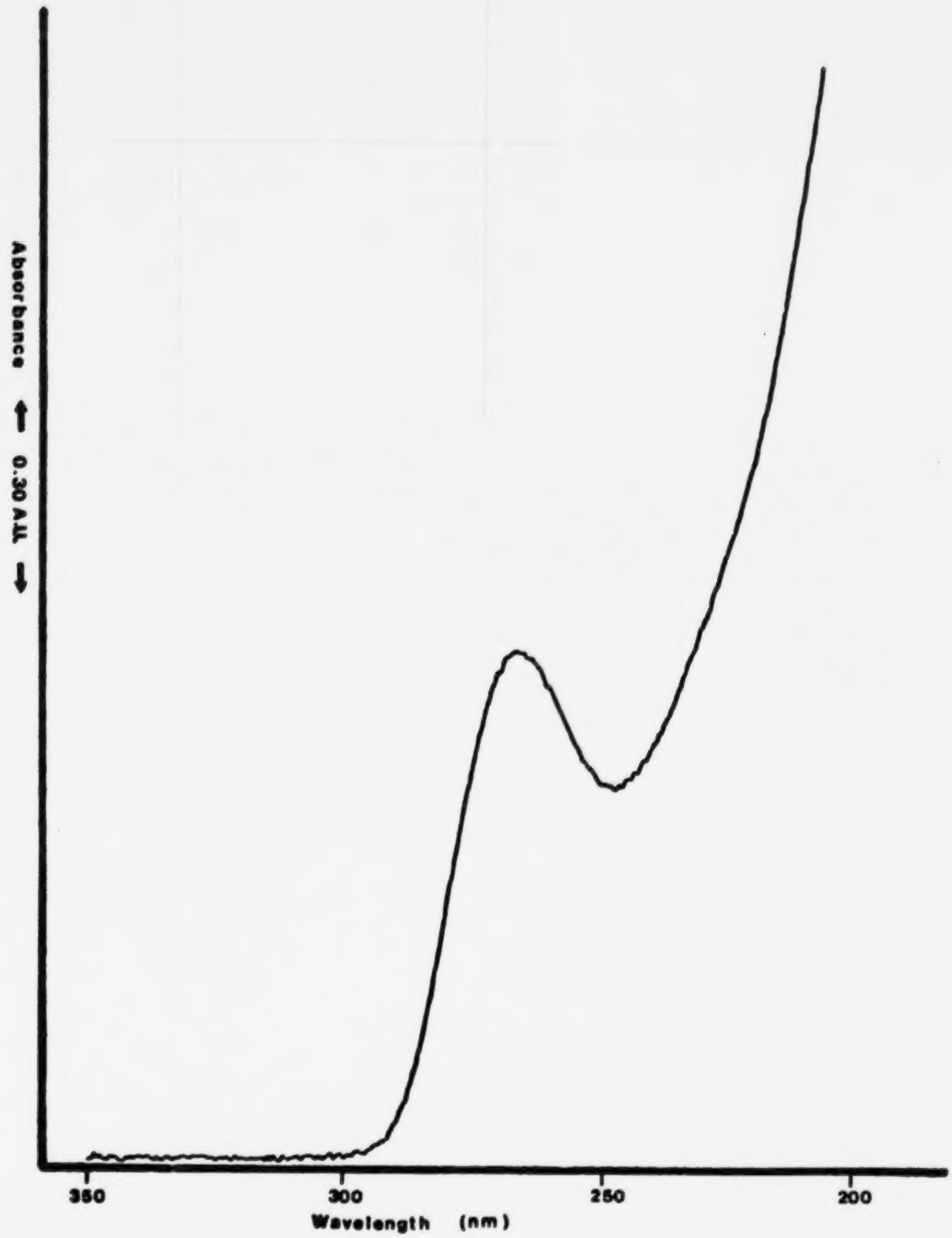


Figure 35. Cytosine (in 0.05 M borate buffer).

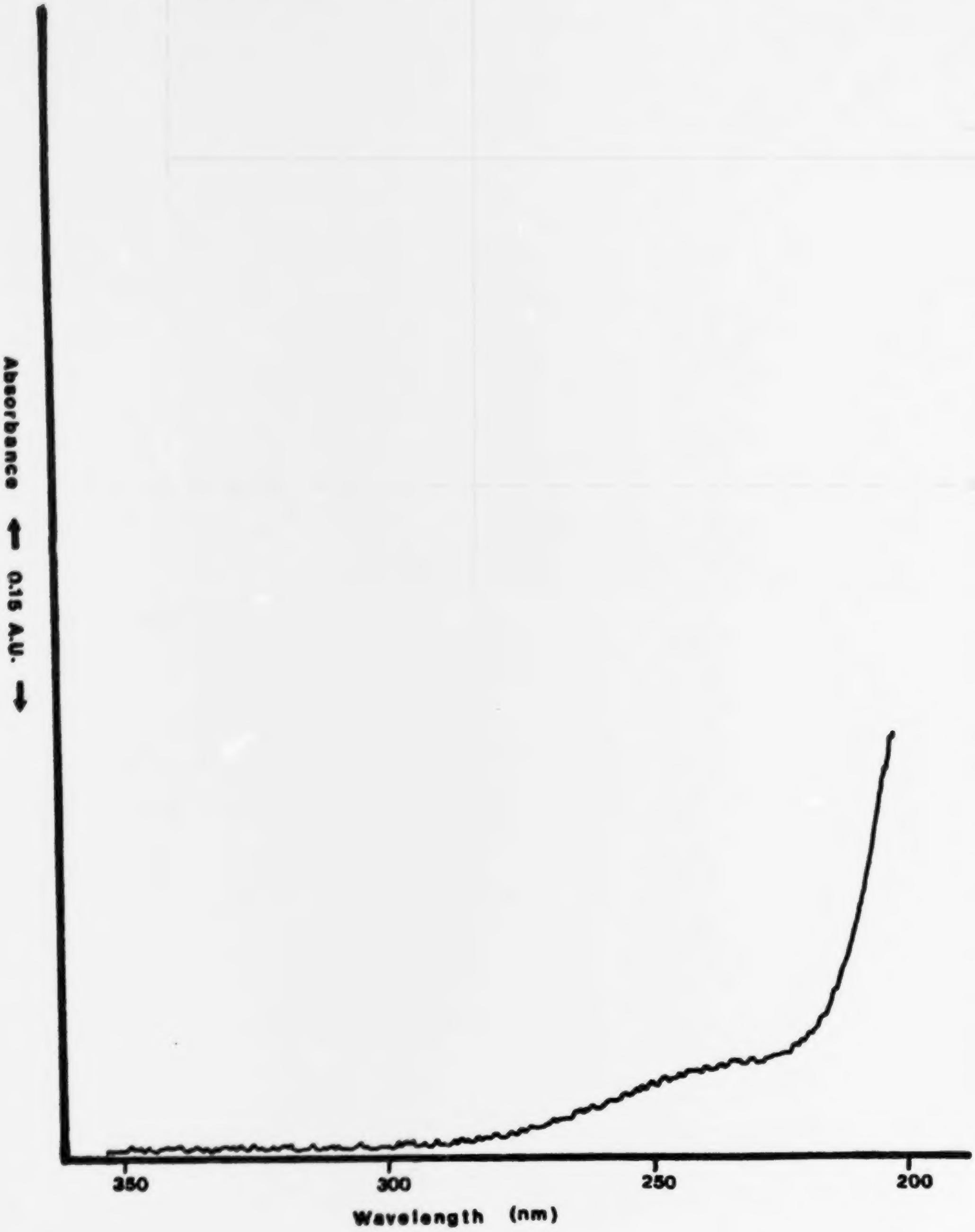


Figure 36. D-glutamic acid (in 0.05 M borate buffer).

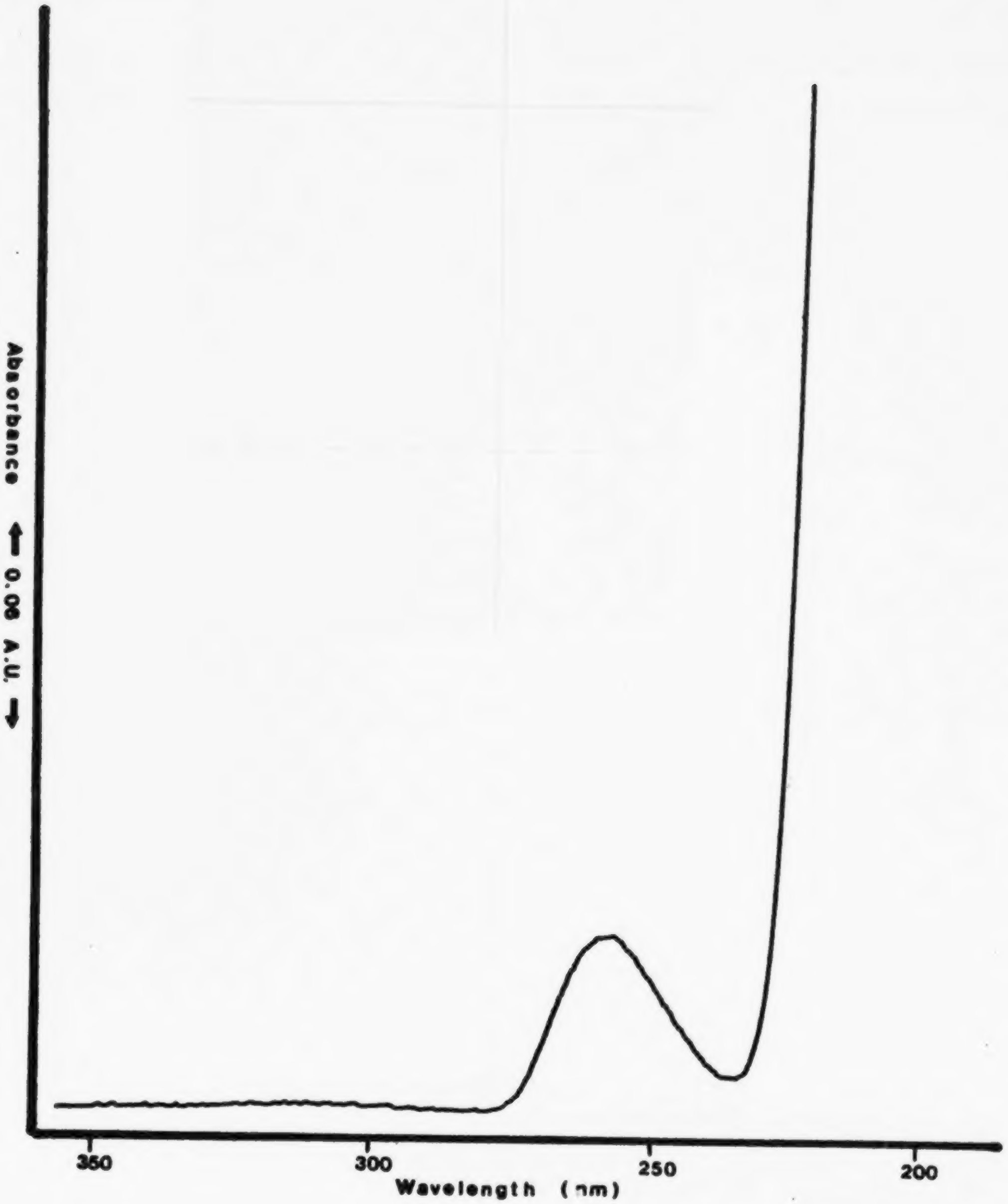


Figure 37. DL-phenylalanine (in 0.05 M borate buffer).

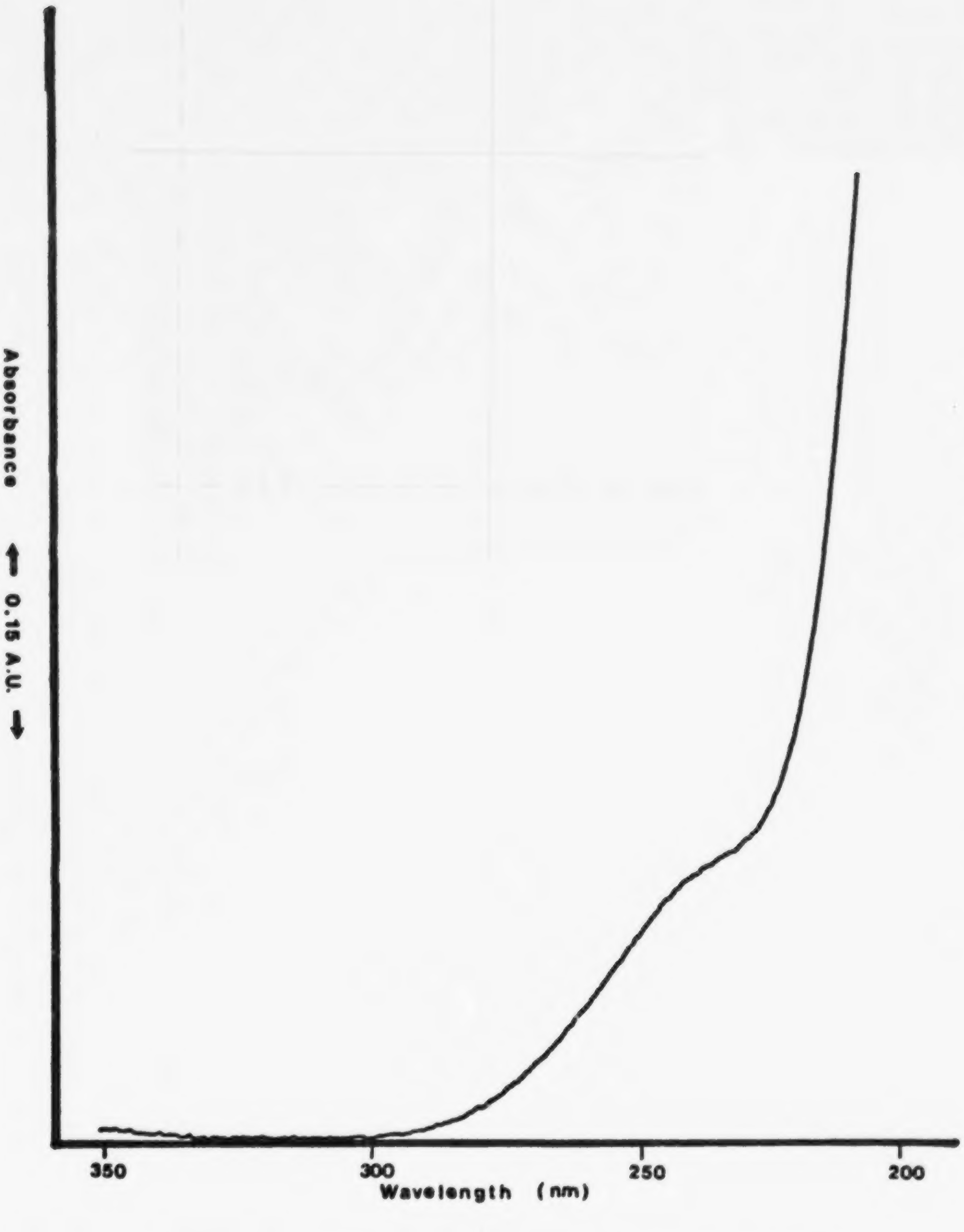


Figure 38. DL-serine (in 0.05 M borate buffer).

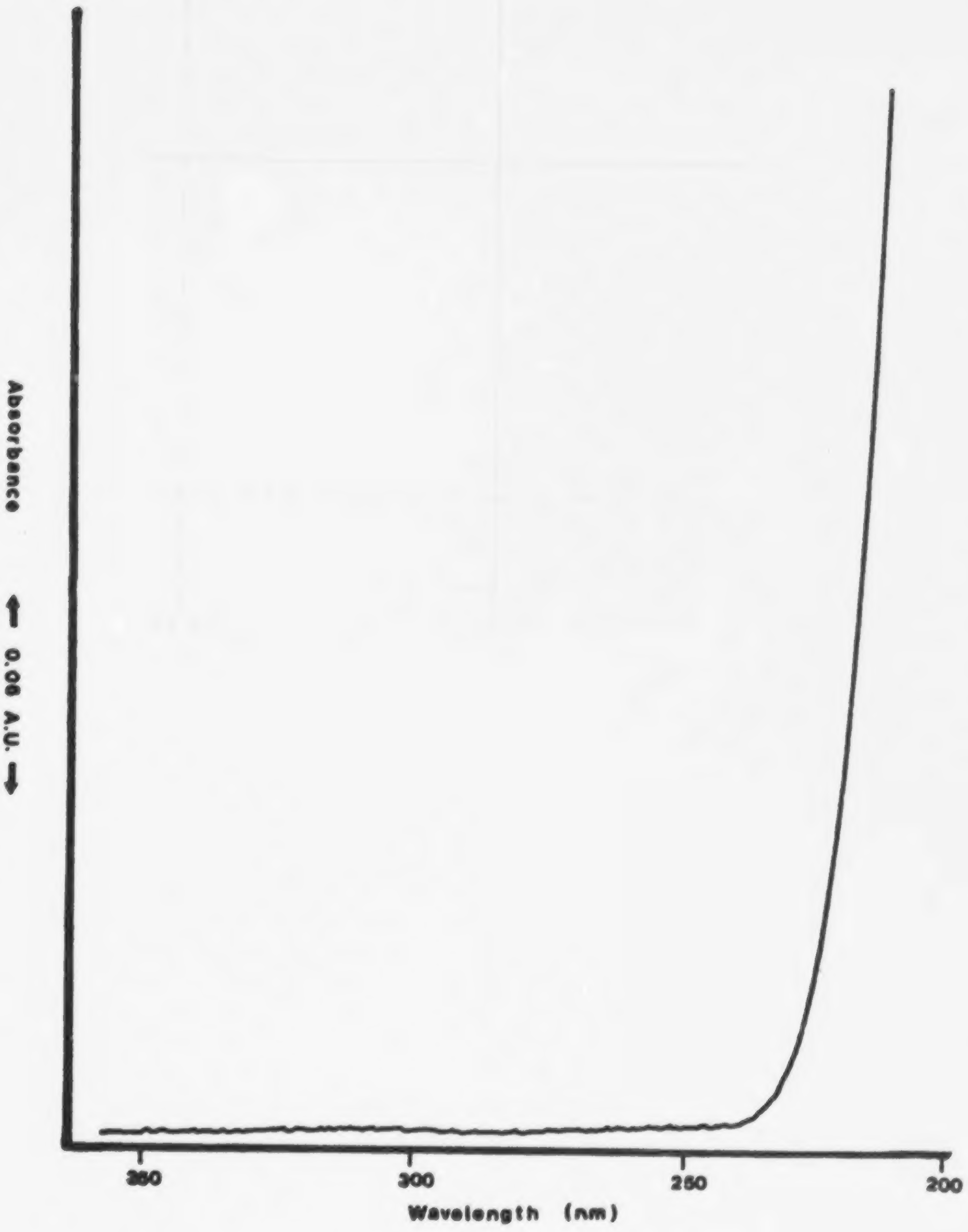


Figure 39. D-valine (in 0.05 M borate buffer).

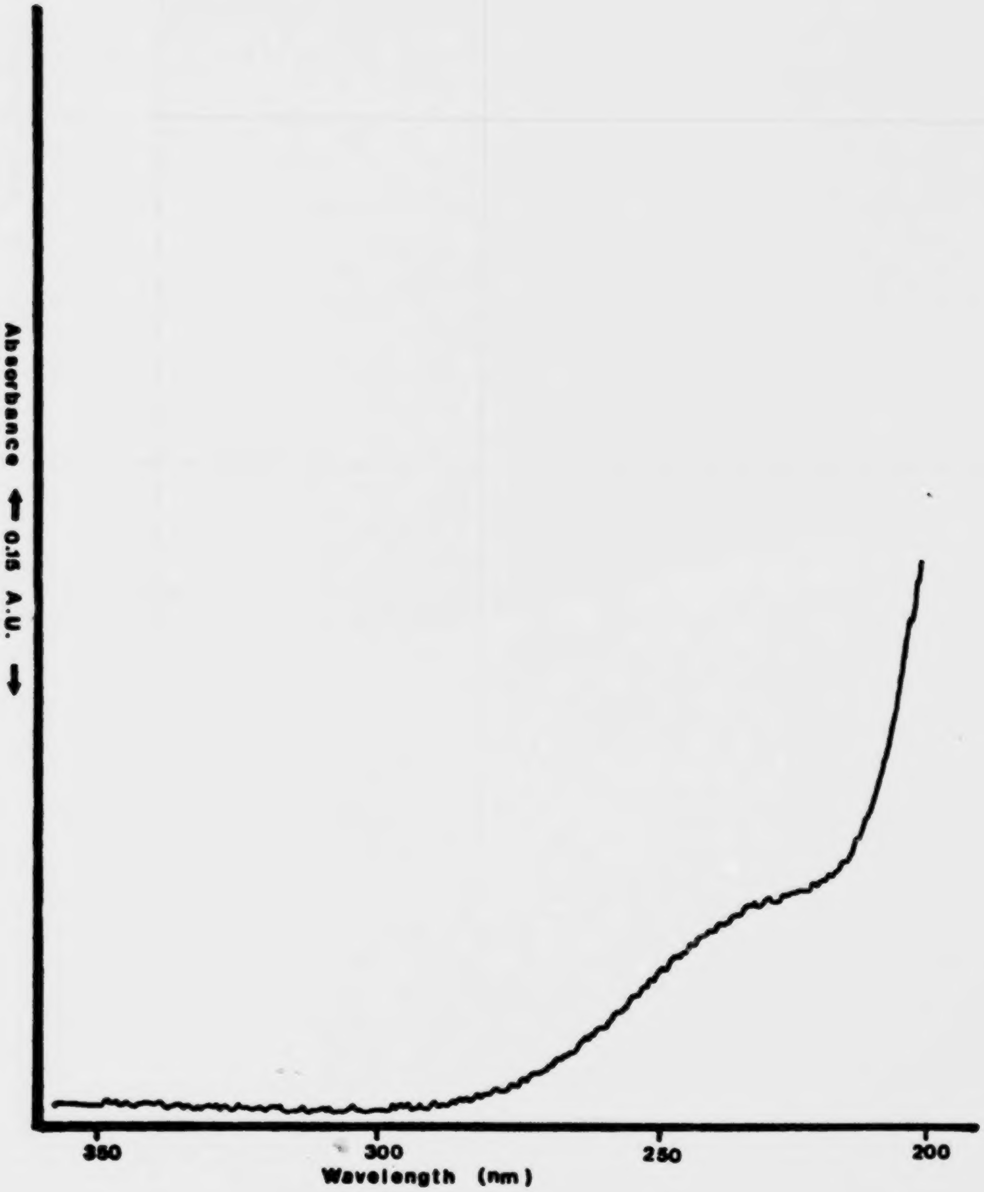


Figure 40. Glycine (in 0.05 M borate buffer).

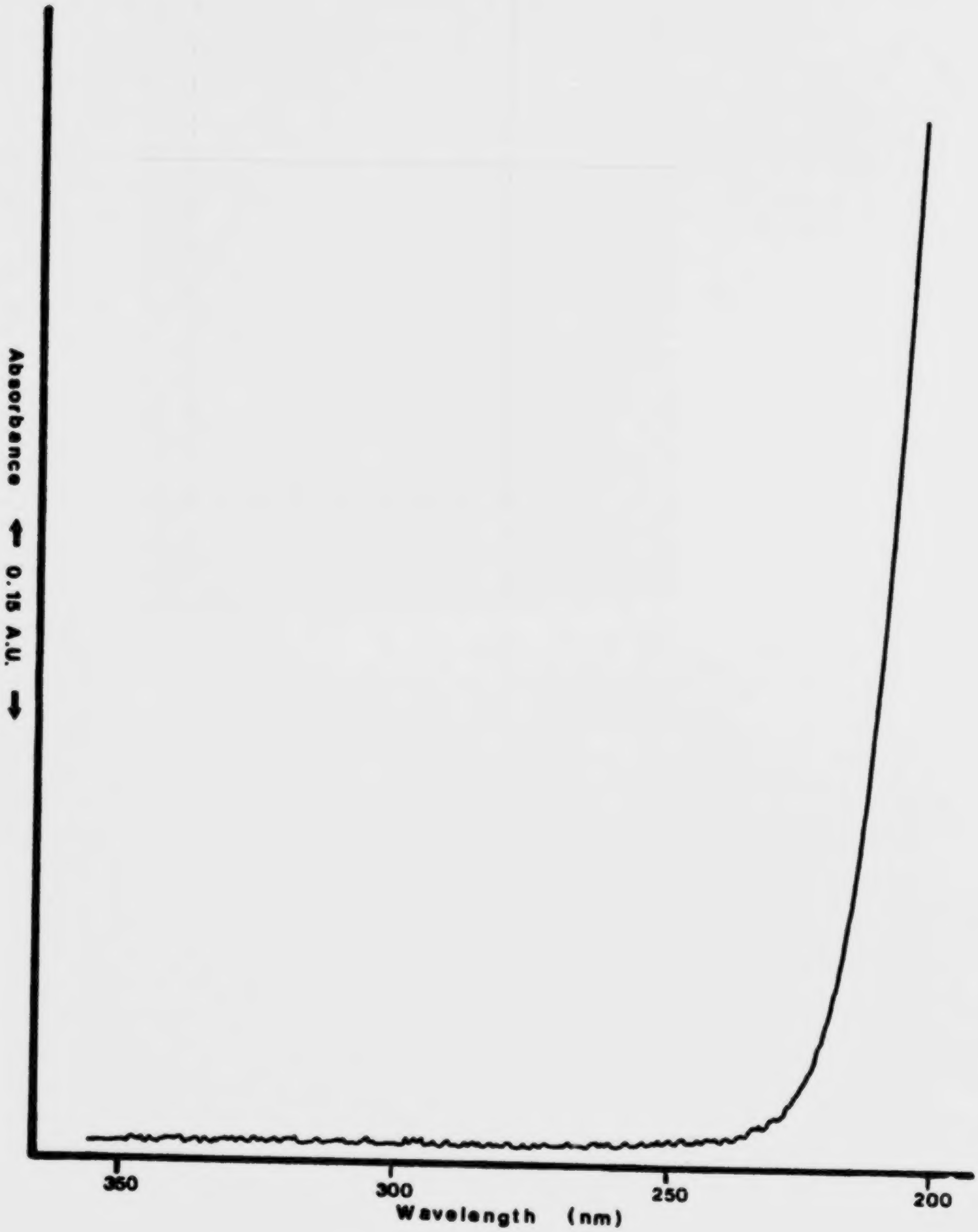


Figure 41. Glycylglycine (in 0.05 M borate buffer).

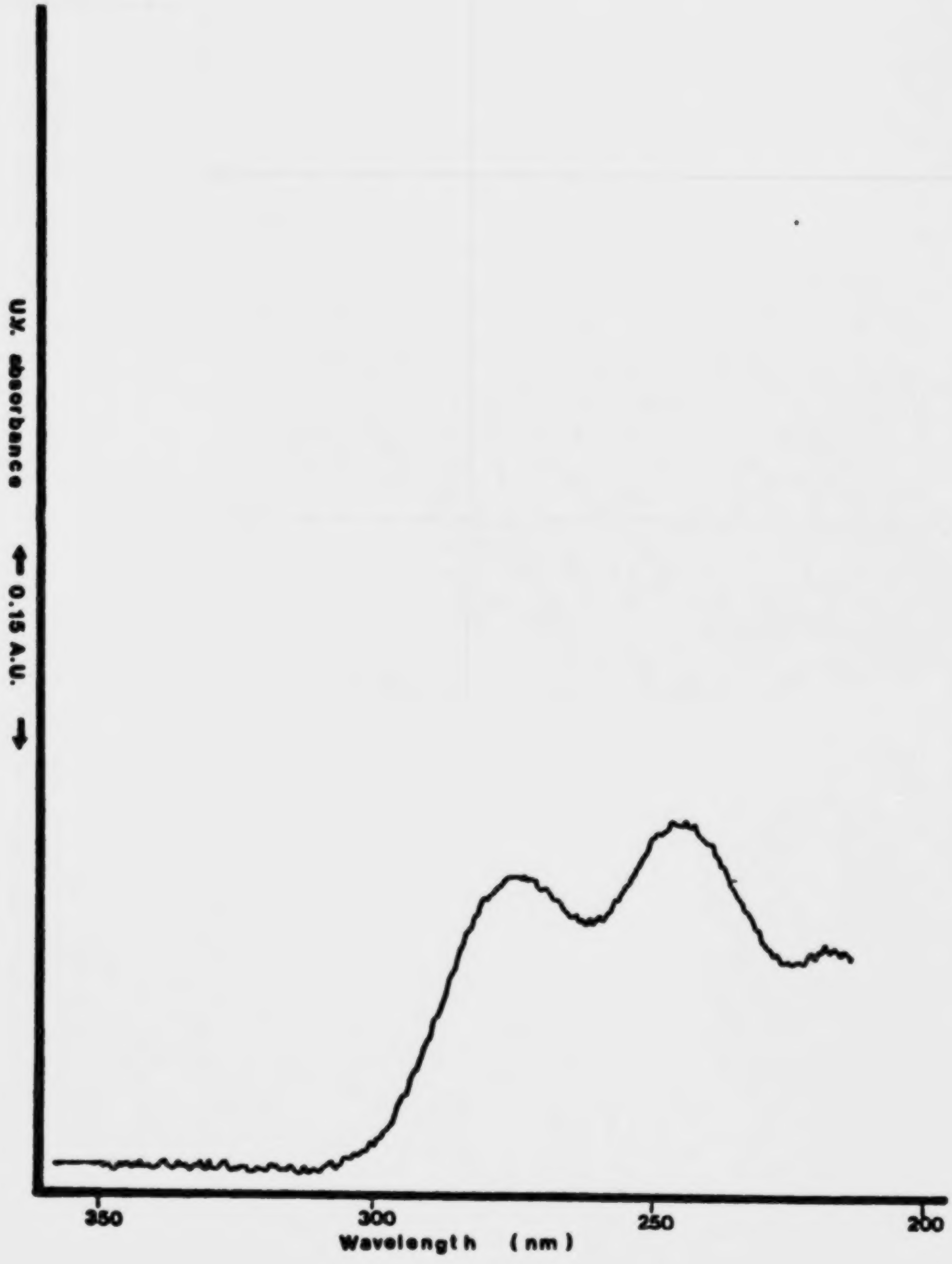


Figure 42. Guanine (in 0.05 M borate buffer).

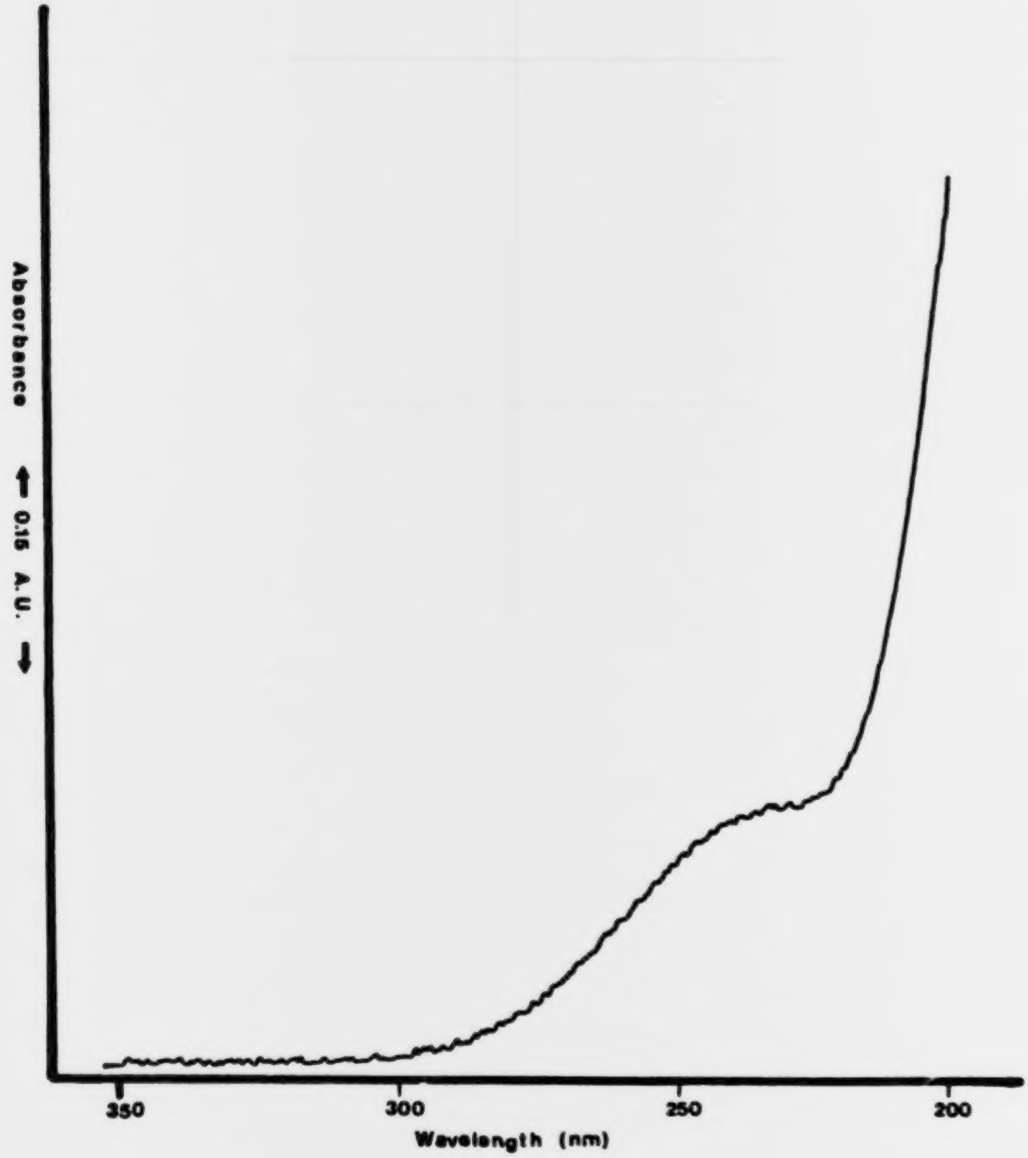


Figure 43. L-(+)-Aspartic Acid (in 0.05 M borate buffer).

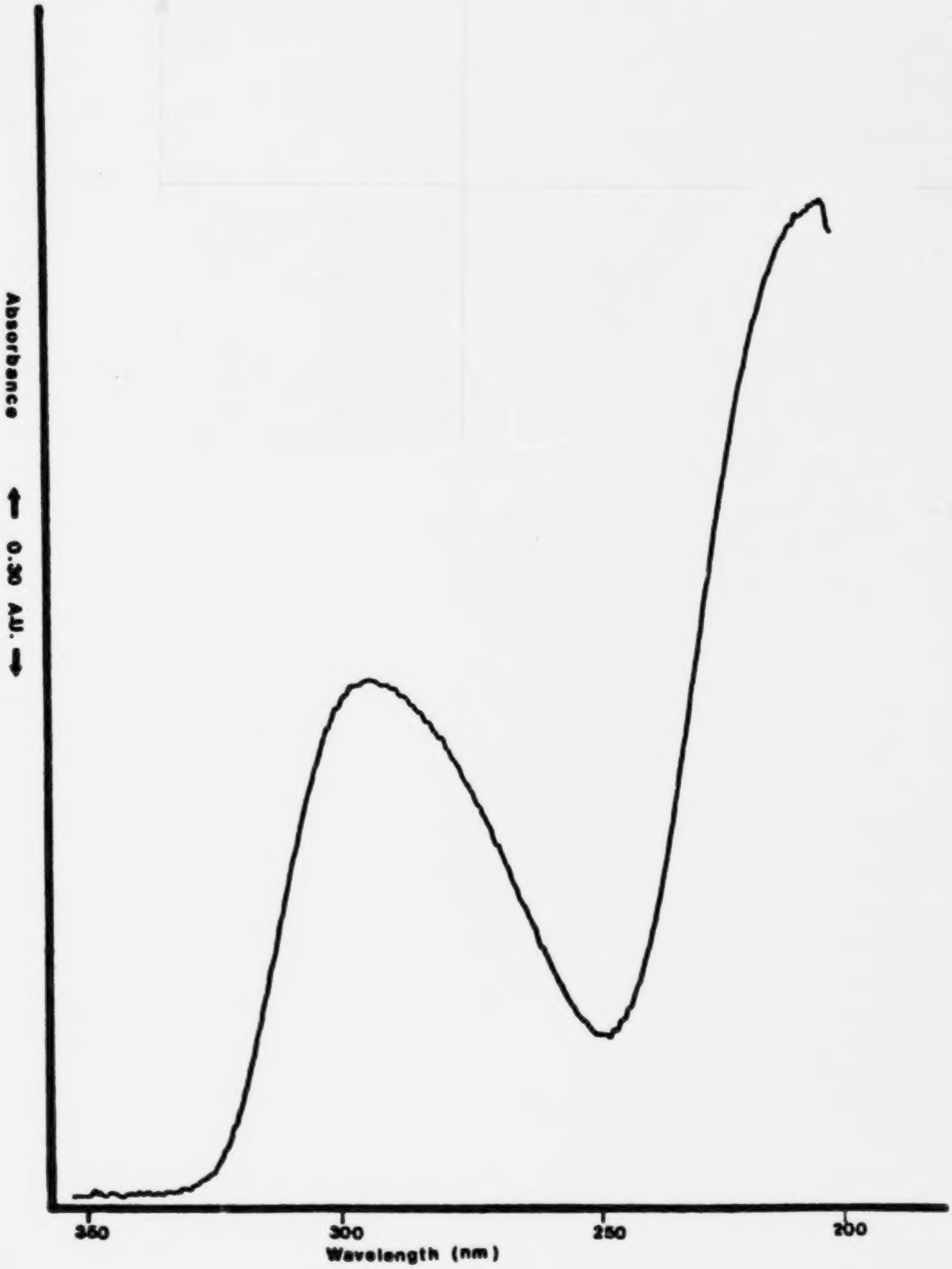


Figure 44. L-(-)-Histidine (in 0.05 M borate buffer).

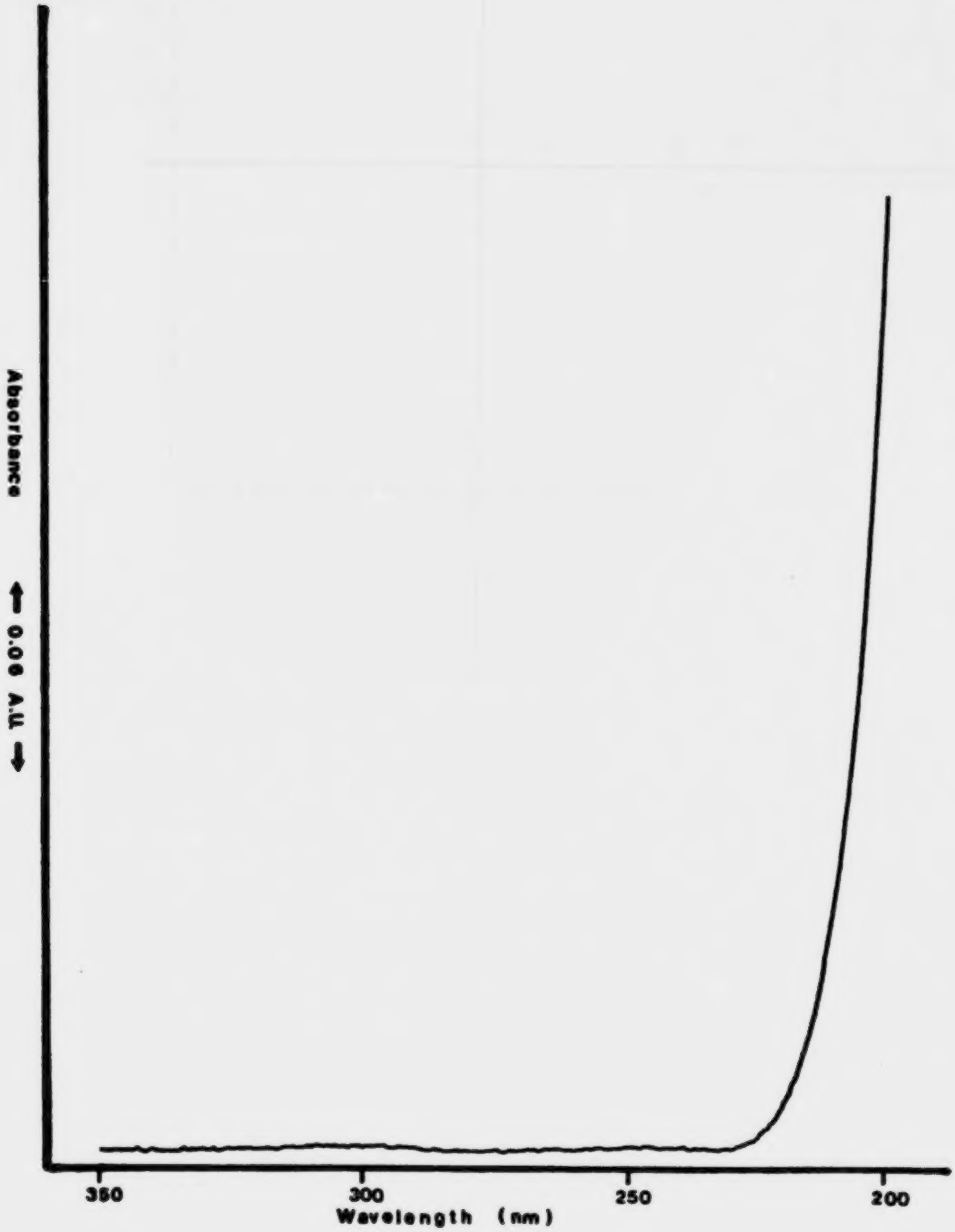


Figure 45. L-(+)-Lysine (in 0.05 M borate buffer).

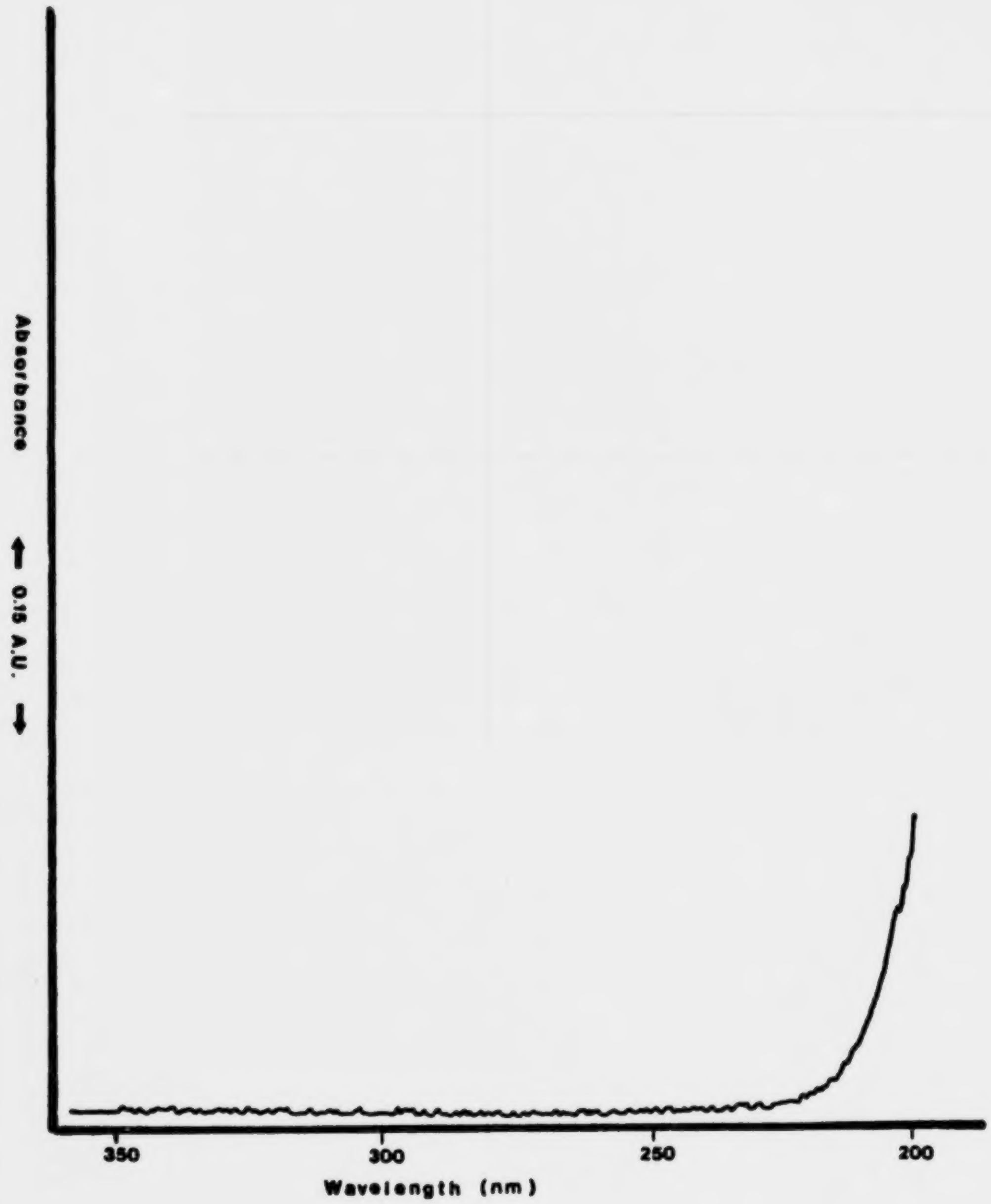


Figure 46. L-(-)-Proline (in 0.05 M borate buffer).

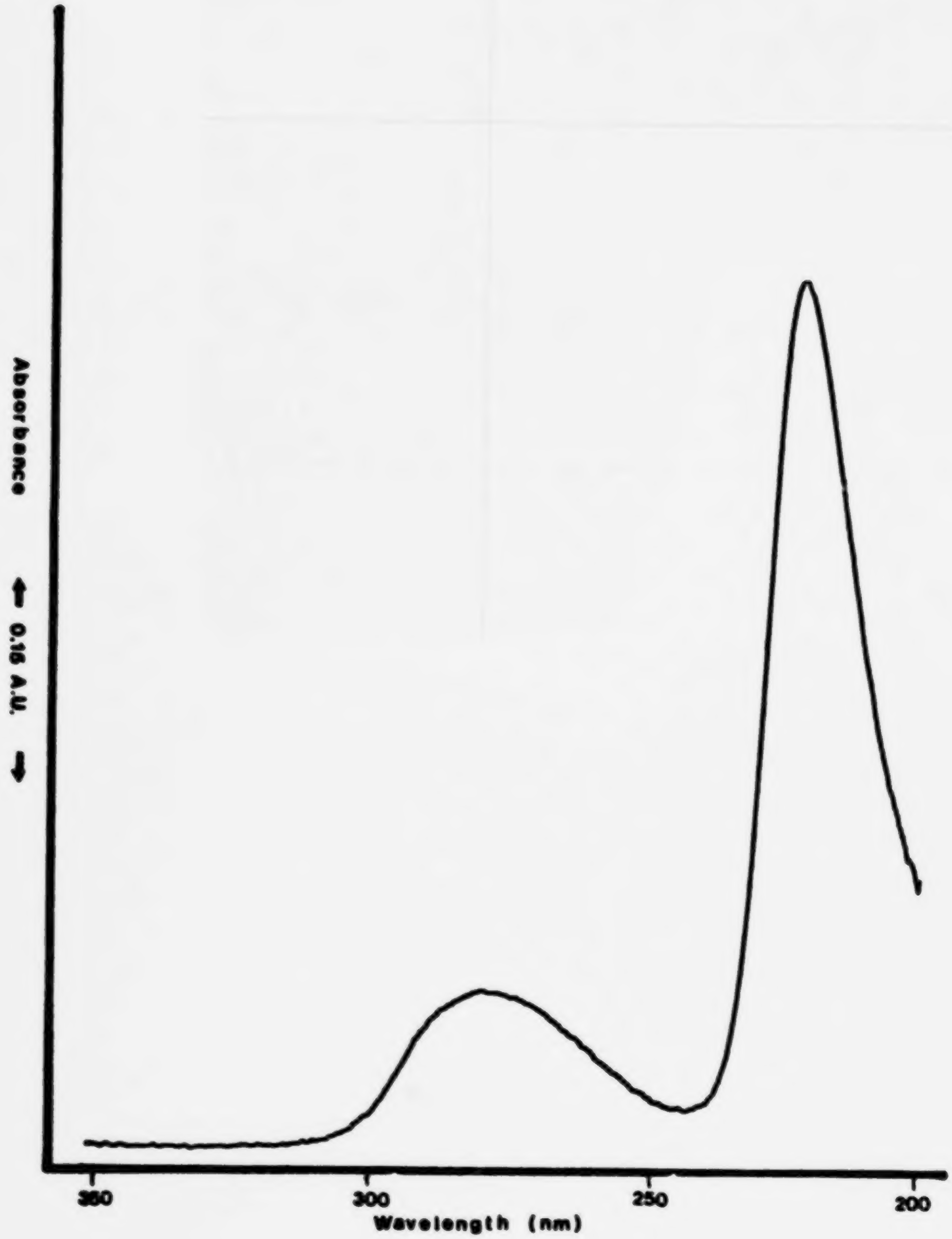


Figure 47. 3-methylindole (skatole) (in 0.05 M borate buffer).

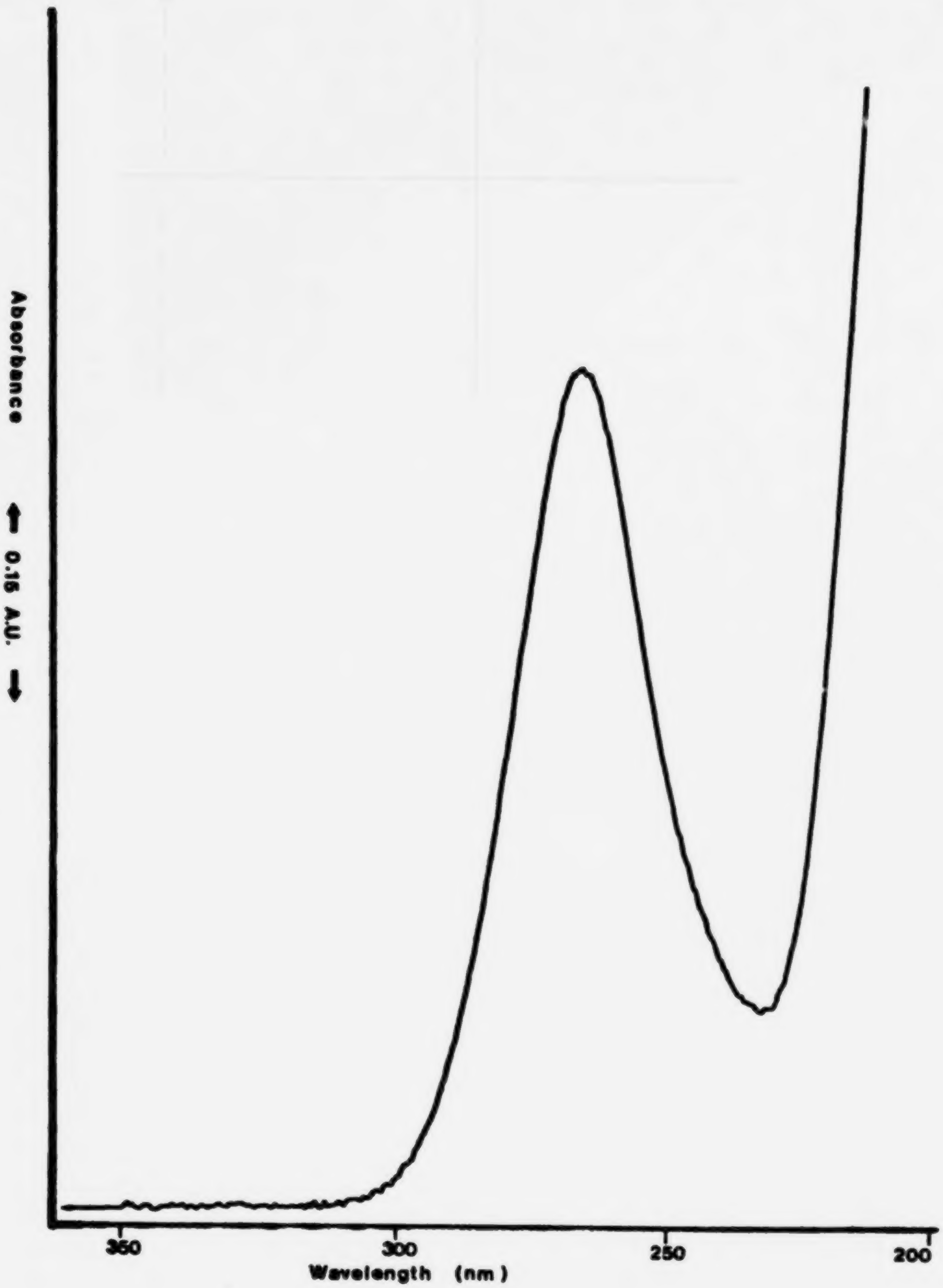


Figure 48. Purine (in 0.05 M borate buffer).

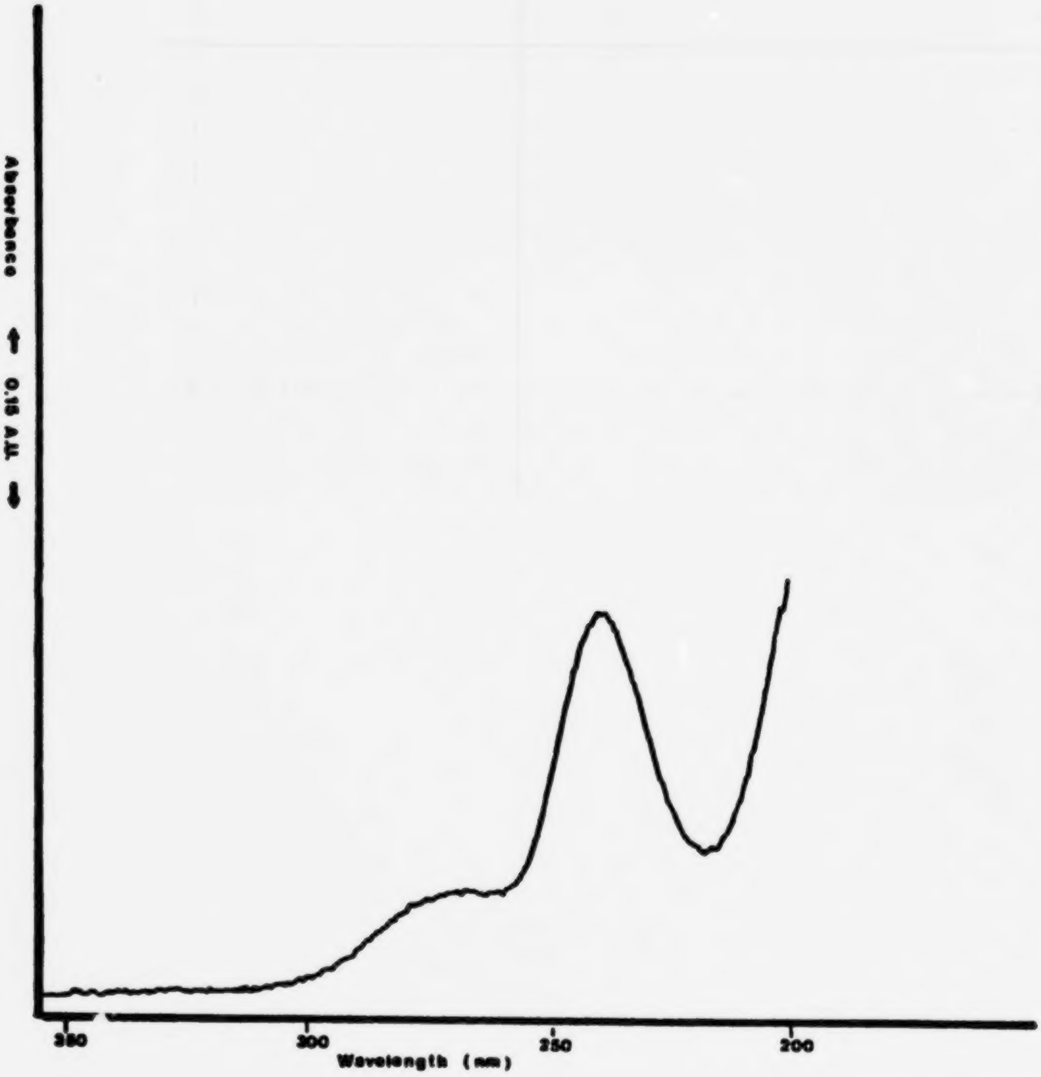


Figure 49. Pyrimidine (in 0.05 M borate buffer).

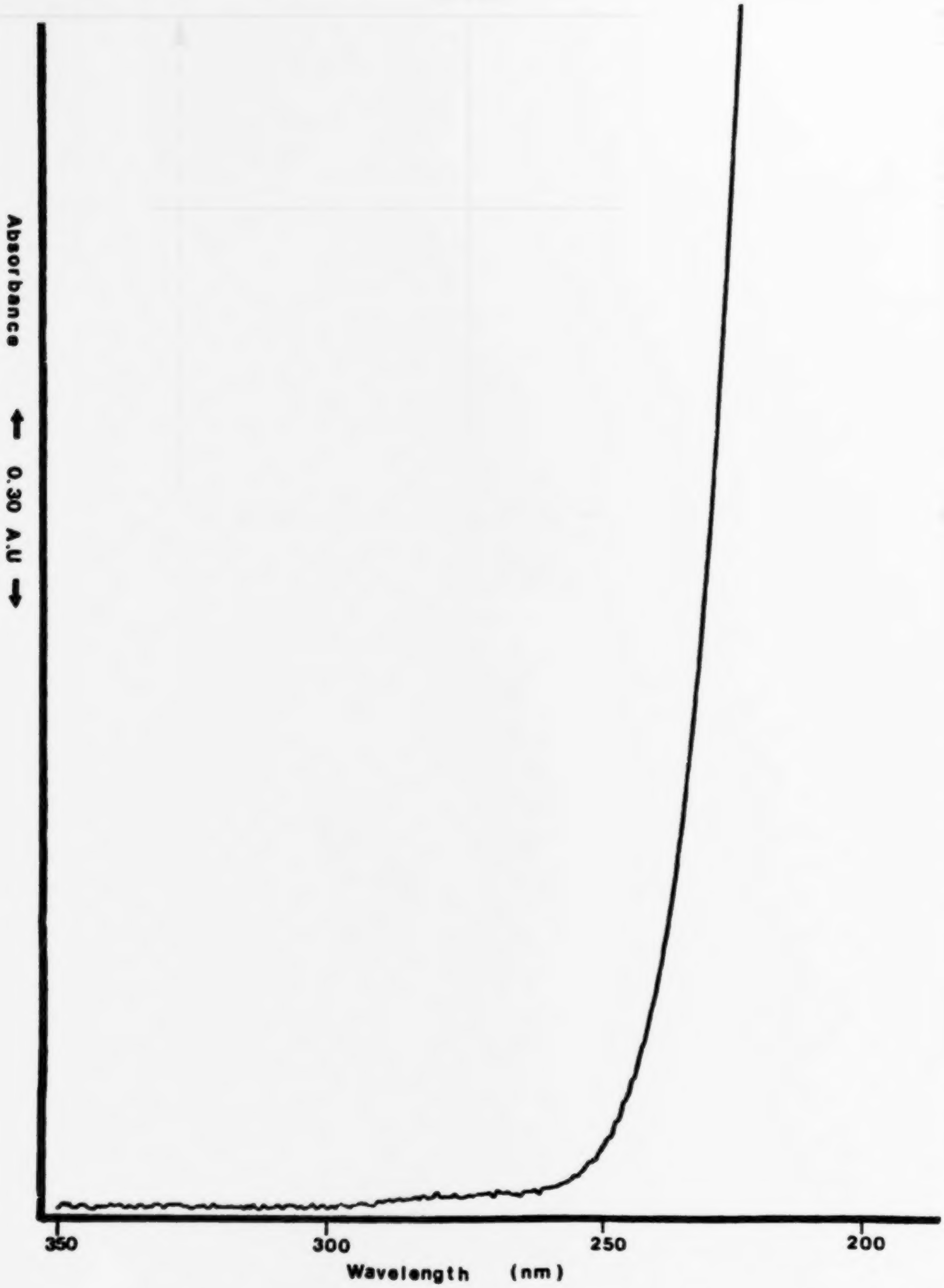


Figure 50. Pyrrole (in 0.05 M borate buffer).

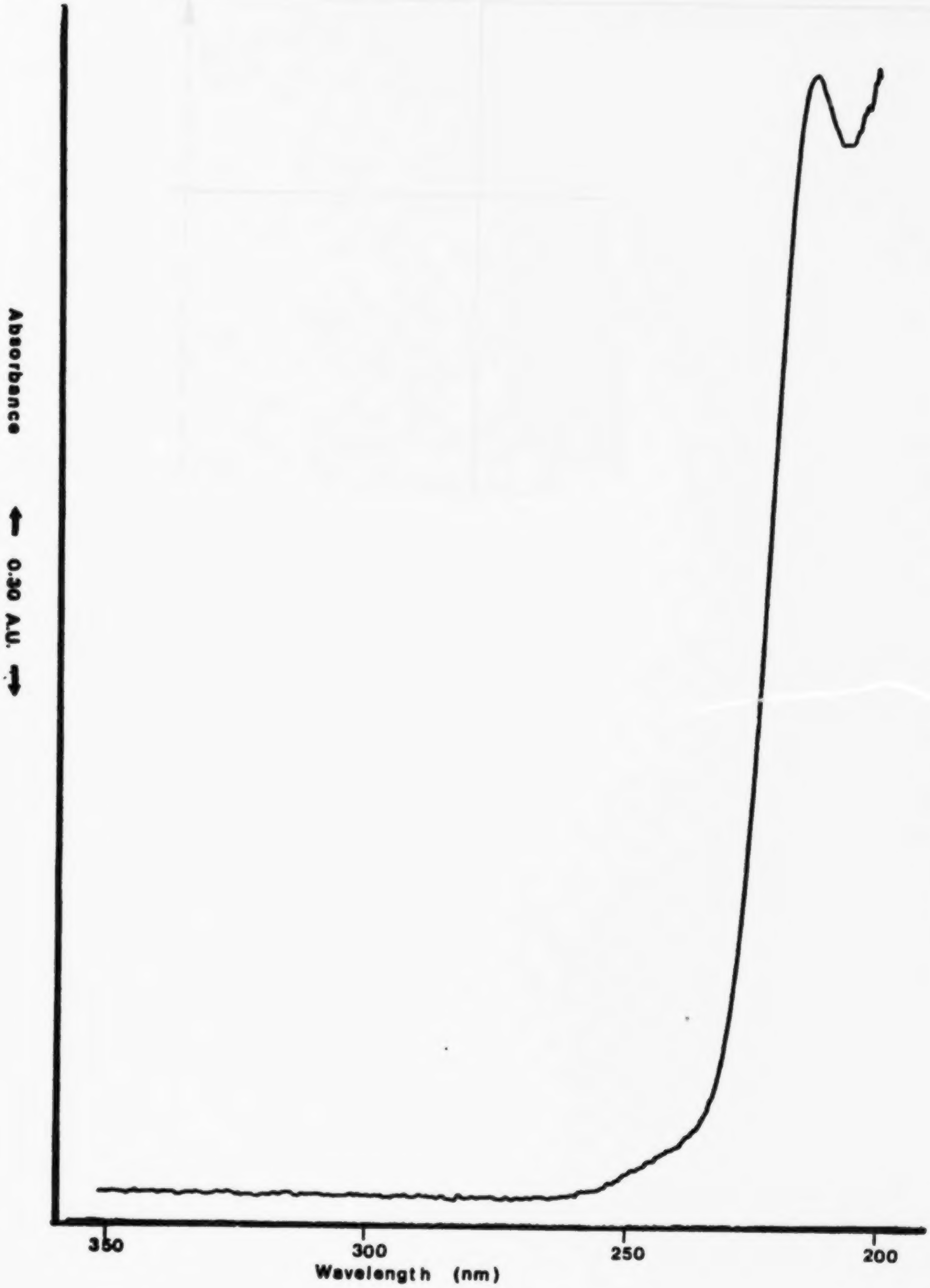


Figure 51. Succinimide (in 0.05 M borate buffer).

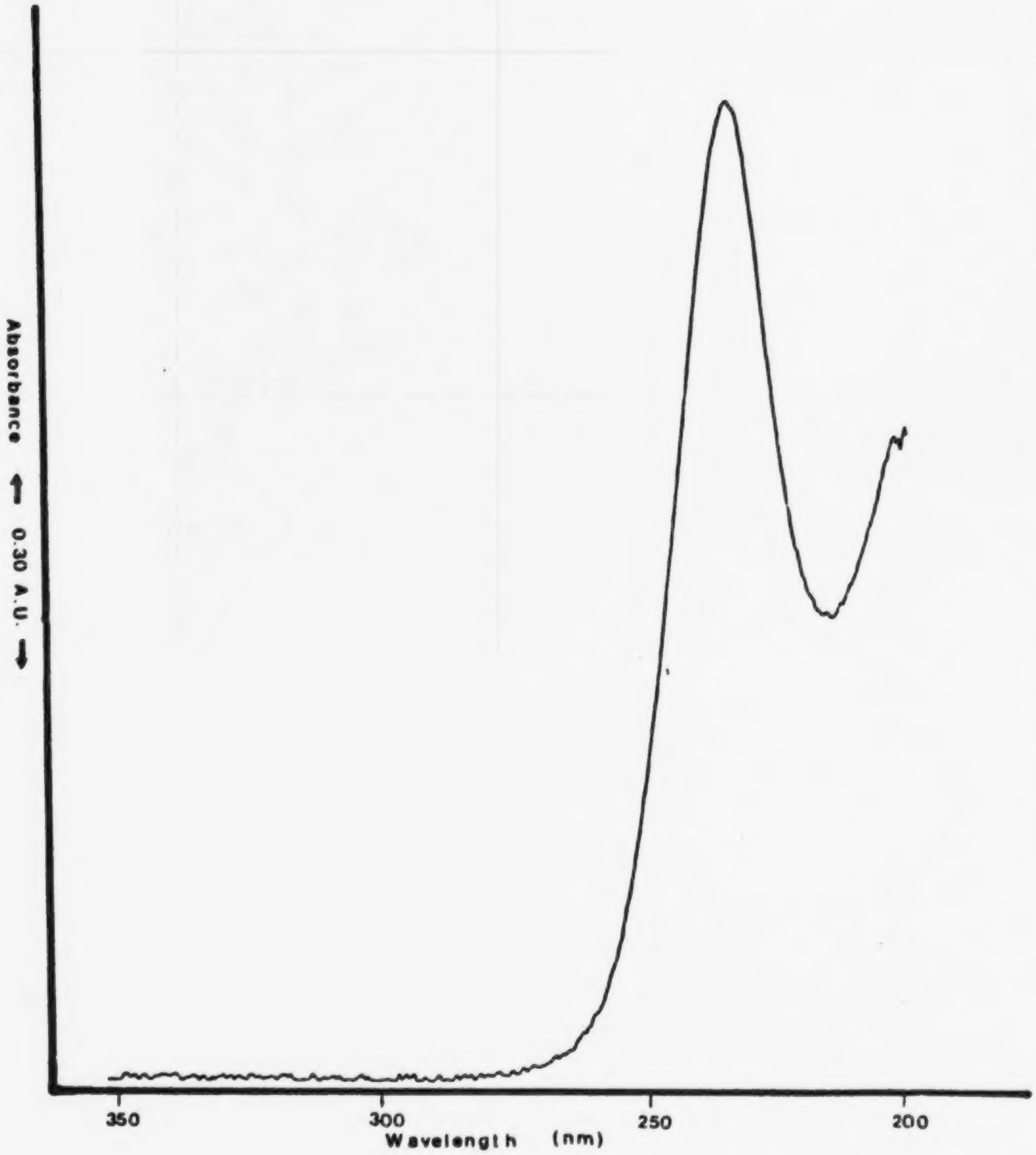


Figure 52. Thiourea (in 0.05 M borate buffer).

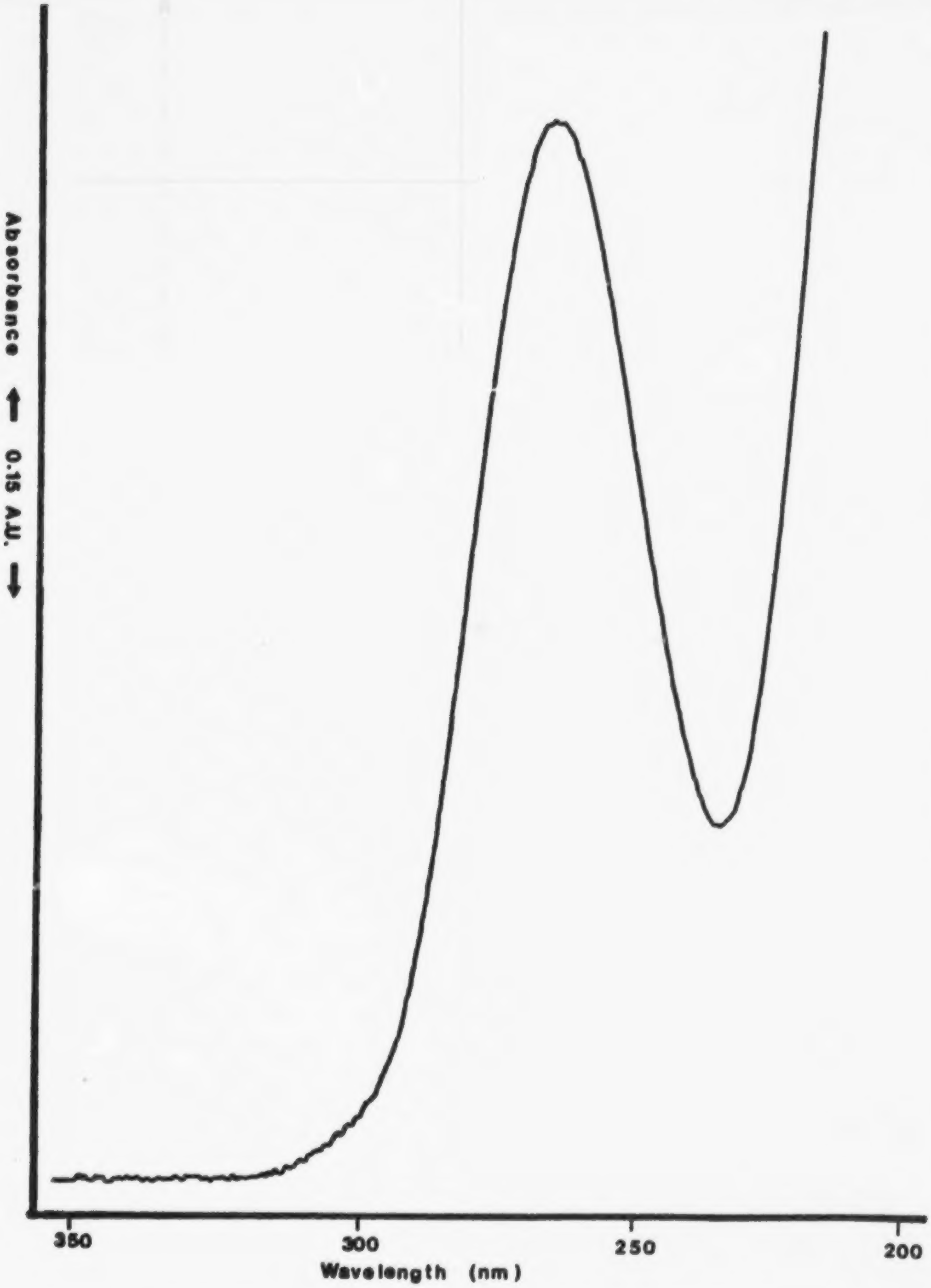


Figure 53. Thymine (in 0.05 M borate buffer).

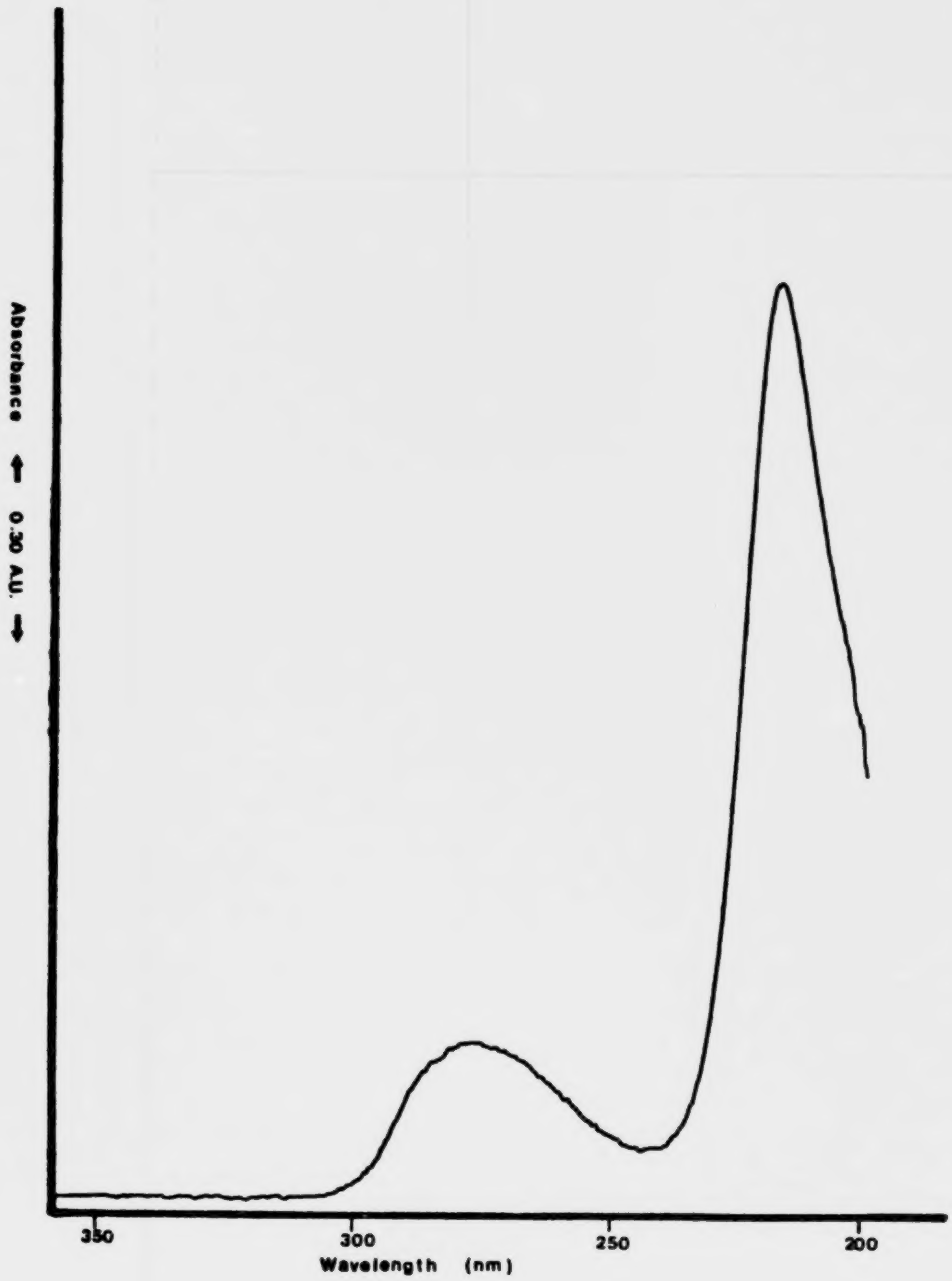


Figure 54. Tryptophan (in 0.05 M borate buffer).

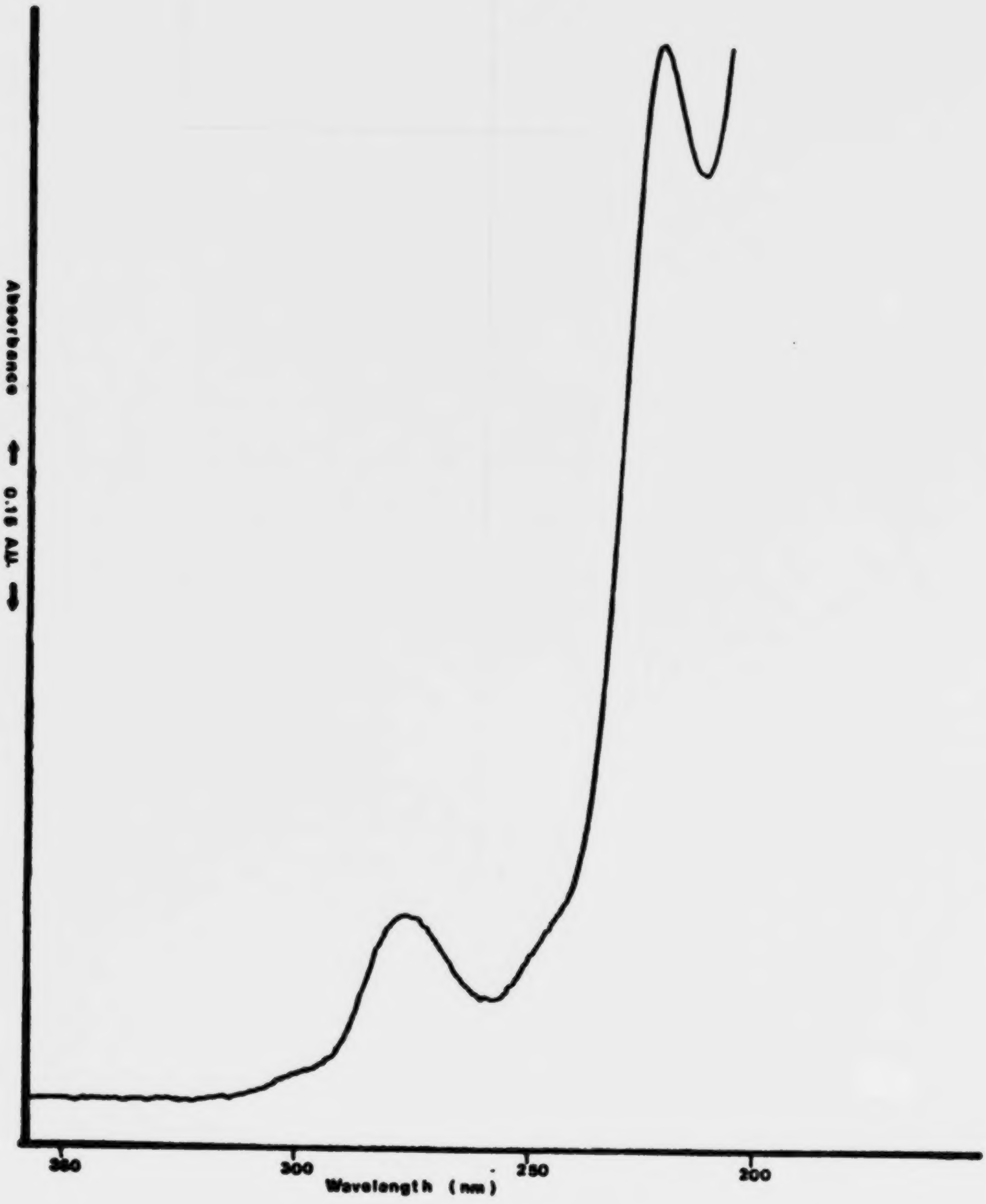


Figure 55. Tyrosine (in 0.05 M borate buffer).

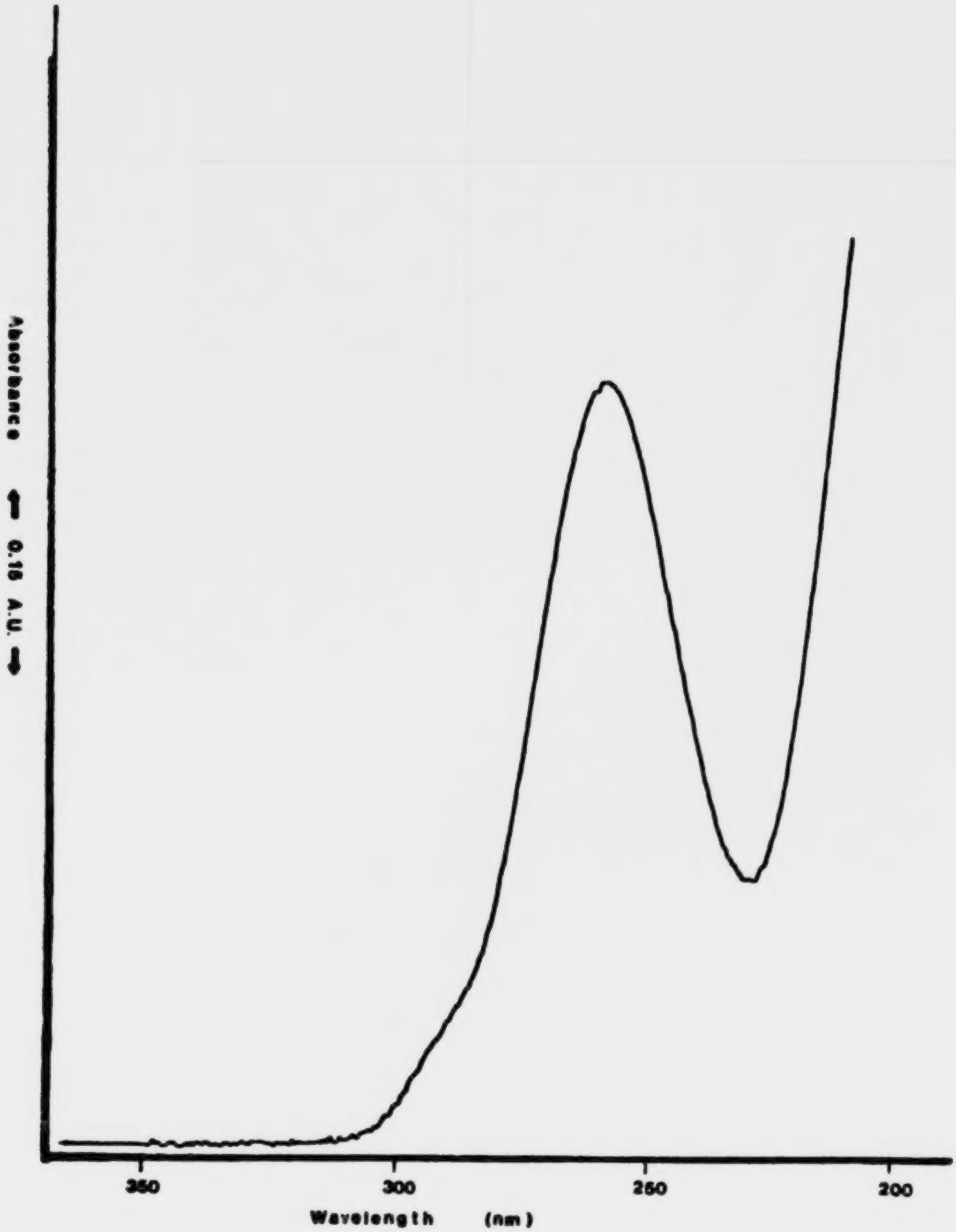


Figure 56. Uracil (in 0.05 M borate buffer).

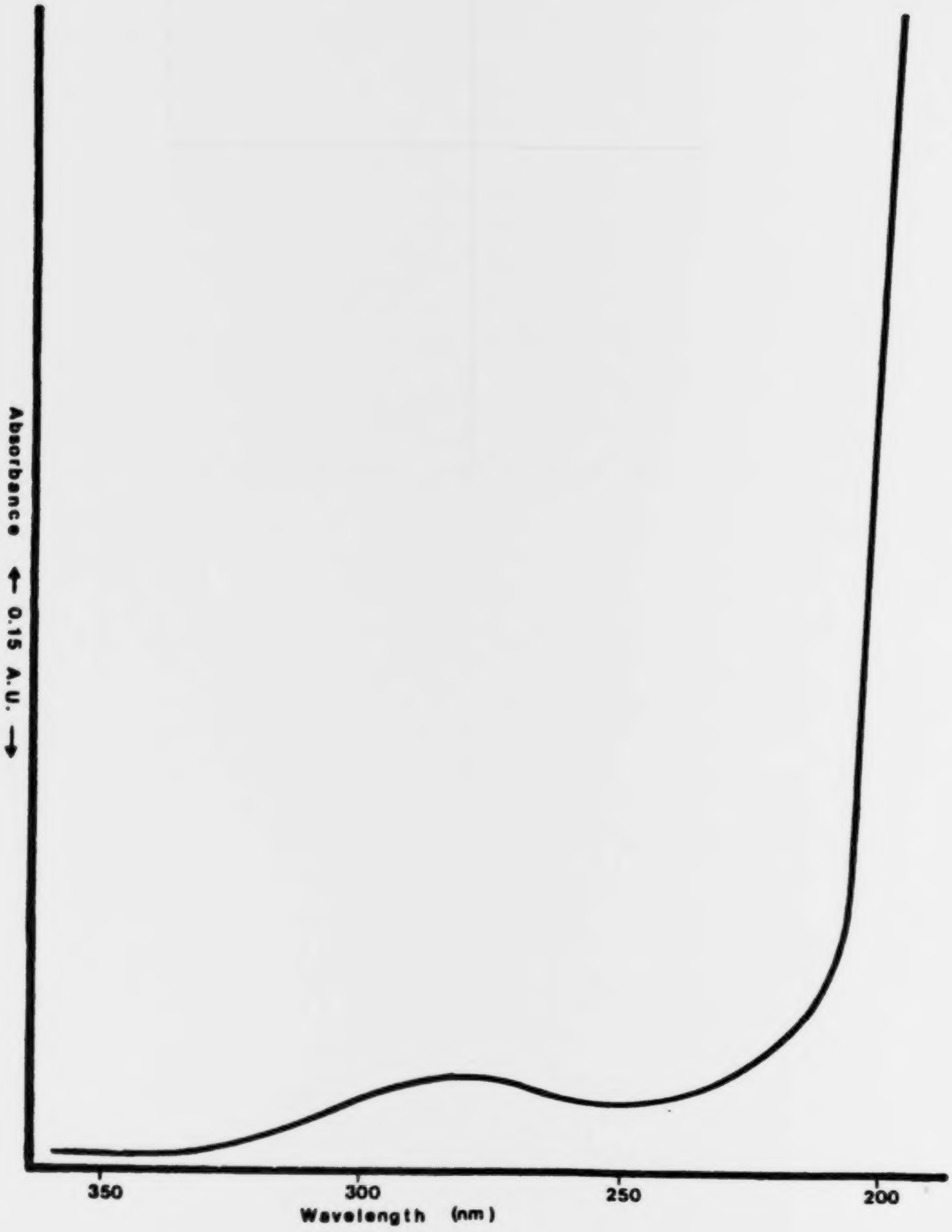


Figure 57. .05 M phosphate buffer, pH = 6.9.

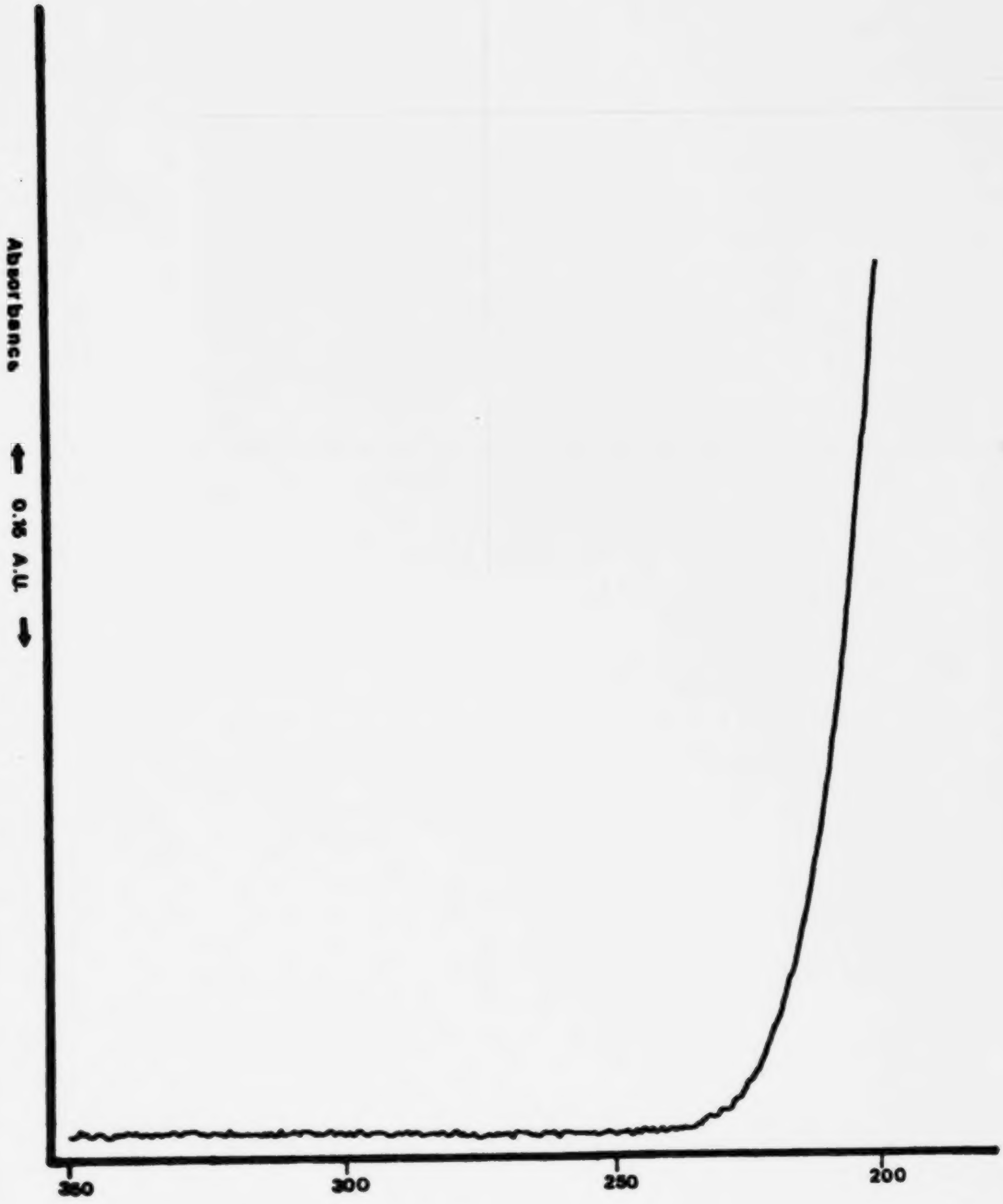


Figure 58. .05 M sodium borate adjusted to pH 8.9 with H_3PO_4.

TABLE 27. SPECTRAL DATA FOR SOME REFERENCE NITROGENOUS COMPOUNDS

compound	position of maximum absorbance (nm)[a]		position of minimum absorbance (nm)		$\frac{\text{absorbance}_{max}}{\text{absorbance}_{min}}$		$\frac{\text{absorbance at 220 nm}}{\text{absorbance at 233 nm}}$	
	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer
adenine	260	260	227	229	4.9	4.0	1.4	1.3
barbituric acid	-	257	-	230	-	4.8	1.2	1.7
5-chlorouracil	276	293	240	248	3.3	3.6	2.9	1.7
creatinine	234	233	217	215	1.6	1.5	0.7	0.8
cytosine	267	266	247	248	1.4	1.4	1.3	1.3
guanine	276 & 250	272 & 243	259	263 & 228	-	-	1.3	1.4
ℓ-histidine	-	293	-	249		3.2	1.7	1.7
purine	265	268	223	233	4.9	4.0	0.8	2.4
pyrimidine	242	240	218	219	4.4	2.9	0.4	2.1
pyrrole	flat	flat	flat	flat	-	-	5.1	-
succinimide	239	211	232	205	5.0	1.07	1.9	-
thymine	265	265	233	235	3.3	2.9	2.2	2.3
tryptophan	279	276	243	243	2.7	3.0	6.0	7.1

(continued)

TABLE 27 (continued)

compound	position of maximum absorbance (nm)[a]		position of minimum absorbance (nm)		$\frac{\text{absorbance}_{\text{max}}}{\text{absorbance}_{\text{min}}}$		$\frac{\text{absorbance at 220 nm}}{\text{absrobance at 233 nm}}$	
	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer	.05 M phosphate buffer	.05 M borate buffer
tyrosine	276 & 222	276 & 222	245	260	7.2	10.3	1.6	2.3
uracil	259	259	228	229	4.0	2.9	1.3	1.3
indole	273 & 217	-	240	-	3.7	-	8.4	-

[a] Underlined values were used in computation of absorbance ratio.

Chromatographic Resolution of Concentrated Natural Samples

Isocratic Elution With U.V. Detection Only--

Chromatograms of concentrated Charles River (Appendix B, Figure B-5), early Concord River (Appendix B, Figure B-6), and Merrimack River (Appendix B, Figure B-7) water samples all contained only large peak eluting rapidly from the Zipax SCX and Zorbax C-8 columns, and occasionally smaller secondary peaks. No distinct peaks characteristic of identifiable nitrogenous compounds were detected. Both Kjeldahl-N (Table 28) and fluorometric analyses (Appendix B, Figures B-8 and B-9) of aliquots taken from the chromatographic column after injection of concentrated field samples, however, indicated the presence of nitrogenous organic compounds beyond the initial large elution peak. The approximate mass of Kjeldahl-N in each aliquot was calculated from the product of the Kjeldahl-N value (mg/L) and the volume analyzed (liters). These data showed that approximately 82% of the nitrogen content of the eluting nitrogenous compounds was found in the first 10 ml of the chromatographic effluent. Some of the higher Kjeldahl-N values occurred in aliquots having corresponding large U.V. absorption. Kjeldahl-N recovieries in other aliquots, however, were observed in low U.V. absorbant portions. Kjeldahl-N values of resolved individual N-organic compounds were probably obscured by the relatively large volume of aliquot required for analysis.

Resolution of some 15 distinct peaks from a concentrated primary sewage effluent collected at Marlboro East sewage treatment facility was achieved using Zorbax C-8 with citric acid, phosphate, and borate buffered eluants of increasing methanol composition. Peak identification was not pursued because of the inability reproducibly to regulate increasing mobile phase strength with the isocratic pump. Separation of this concentrated sample using Zorbax CN (1-octanesulfonic acid eluant), Zipax SCX ($NH_4H_2PO_4$; pH 2.5 eluant) and Aminex A-27 (sodium acetate-acetic acid buffer; pH 4.4 eluant) resulted in the resolution of only 5, 5, and 4 peaks, respectively. Gradient elution would most likely have resulted in improved resolutions.

The ability to resolve several complex mixtures on Zipax SCX using $NH_4H_2PO_4$ (pH 2.5) eluant was also investigated. Urine samples were resolved into six U.V. detectable (233 nm) constituent peak while urochrome constituents in urine and in secondary sewage effluent were resolved into only two large peaks in each case.

Gradient Elution with U.V. and Fluorescence Detection--

The gradient device significantly improved the chromatographic analysis of concentrated field samples over conventional isocratic elution. Chromatograms of concentrated samples resolved on the Zorbax C-8 column using the phosphate to methanol, or borate to methanol gradient elution operating conditions, displayed from 1, to over 40 distinct and reproducible peaks. The Zorbax C-8 column exhibited greater selectivity over the Zipax SCX column and was therefore used exclusively in analyzing the concentrated samples. Nitrogenous compounds in 8 of the 12 samples concentrated by the low temperature distillation-lyophilization method were characterized (Figures 59 to 82).

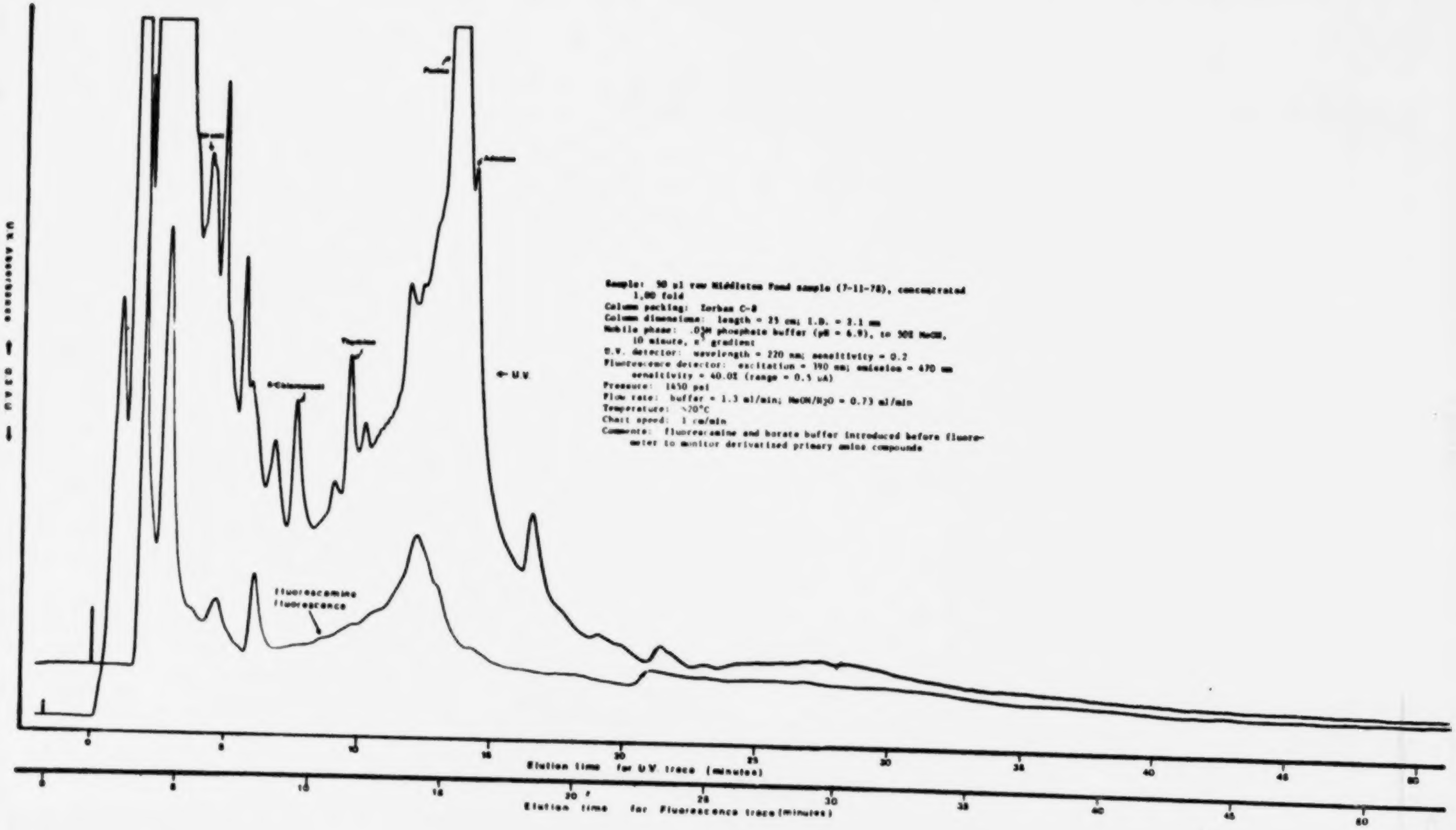
TABLE 28. NITROGEN VALUES IN ALIQUOTS TAKEN FROM THE CHROMATOGRAPHIC EFFLUENT OF ZIPAX SCX AFTER INJECTION OF CONCENTRATED (200 FOLD) MARLBORO WEST POST CHLORINATED EFFLUENT

sample aliquot (ml)	volume analyzed[a] (ml)	Kjeldahl-N[b] (mg/L)	approximate mass of Kjeldahl-N in volume analyzed[c] (mg)	percent Kjeldahl-N in aliquot
0-2	1	7.7	0.77×10^{-2}	33
2-4	1	1.2	0.12×10^{-2}	5
4-8	2	2.8	0.55×10^{-2}	24
8-10	1	4.7	0.47×10^{-2}	20
10-12	1	0.9	0.09×10^{-2}	4
12-14	1	1.6	0.16×10^{-2}	7
14-18	2	0.9	0.18×10^{-2}	8
0.05 ml concentrated sample	.05	354	1.77×10^{-2}	-

[a] Volume analyzed = 1/2 sample aliquot volume. Aliquots were divided equally for separate NH_3 and Kjeldahl-N analysis.

[b] Kjeldahl-N = organic-N since NH_3-N = 0

[c] Mass Kjeldahl-N = concentration of Kjeldahl-N (mg/L) x volume analyzed (liters)



Sample: 50 µl raw Middleton Pond sample (7-11-78), concentrated 1,000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .03M phosphate buffer (pH = 6.9), to 50% MeOH, 10 minute, x^2 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 0.2
Fluorescence detector: excitation = 390 nm; emission = 470 nm sensitivity = 40.0% (range = 0.5 uA)
Pressure: 1650 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds

Figure 59. Chromatogram of raw concentrated Middleton Pond sample (7/11/78), phosphate buffered eluant, with fluorescamine derivatized fluorescence.

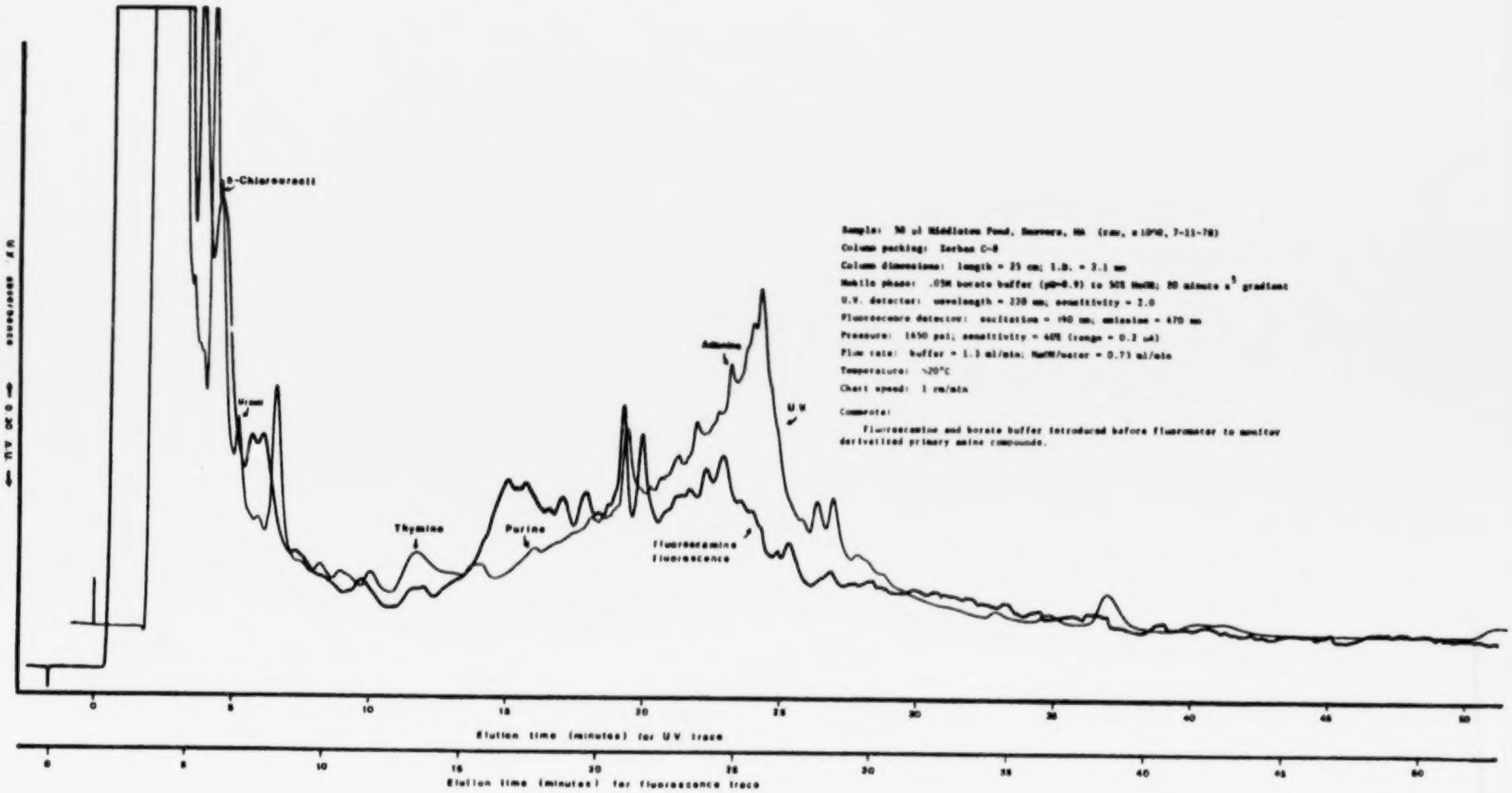


Figure 60. Chromatogram of raw concentrated Middleton Pond sample (7/11/78), borate bufferend eluant, with fluorescamine derivatized fluorescence.

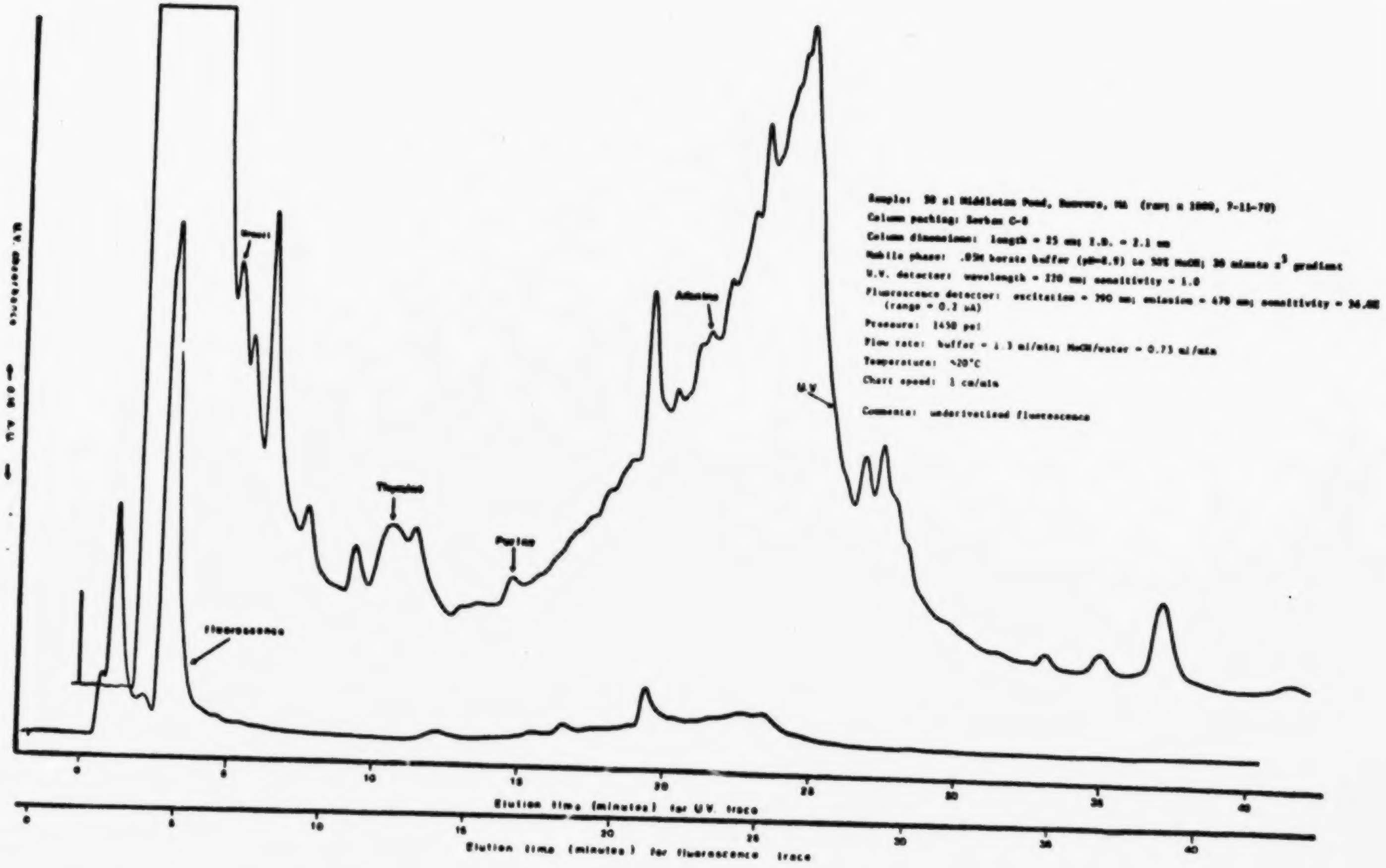


Figure 61. Chromatogram of raw concentrated Middleton Pond sample (7/11/78), borate buffered eluant, with underivatized fluorescence.

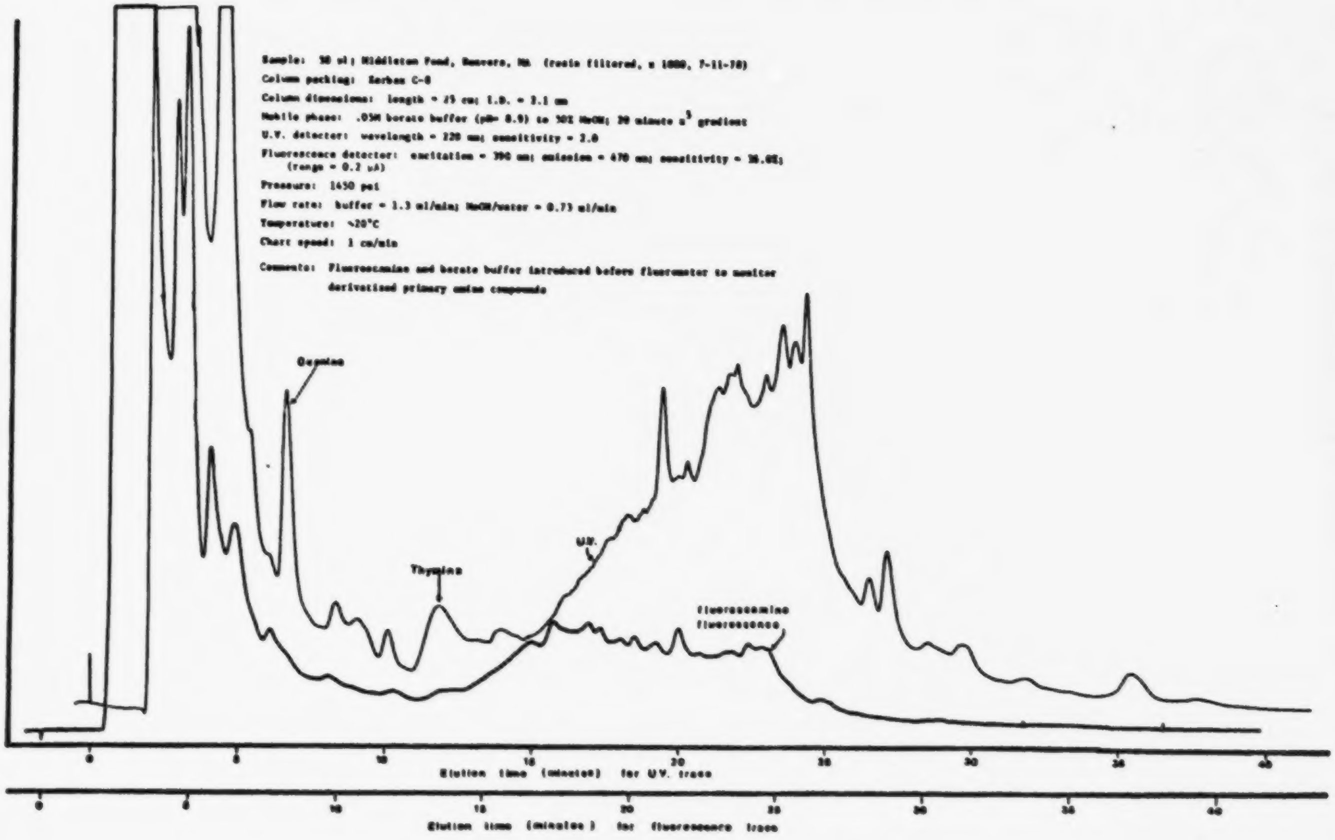


Figure 62. Chromatogram of resin-filtered, concentrated Middleton Pond sample (7/11/78), borate buffered eluant, with fluorescamine derivatized fluorescence.

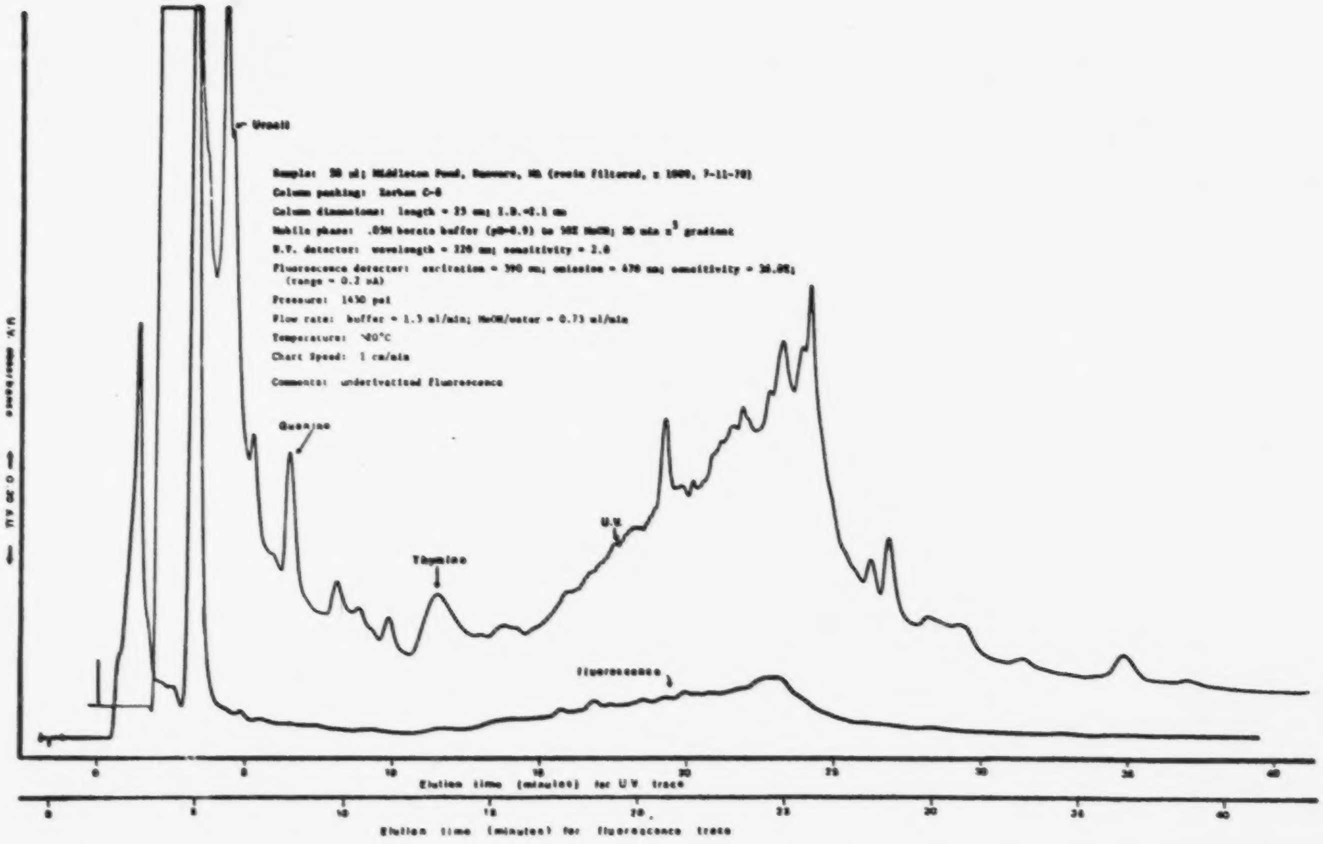


Figure 63. Chromatogram of resin-filtered, concentrated Middleton Pond sample (7/11/78), borate buffered eluant, with underivatized fluorescence.

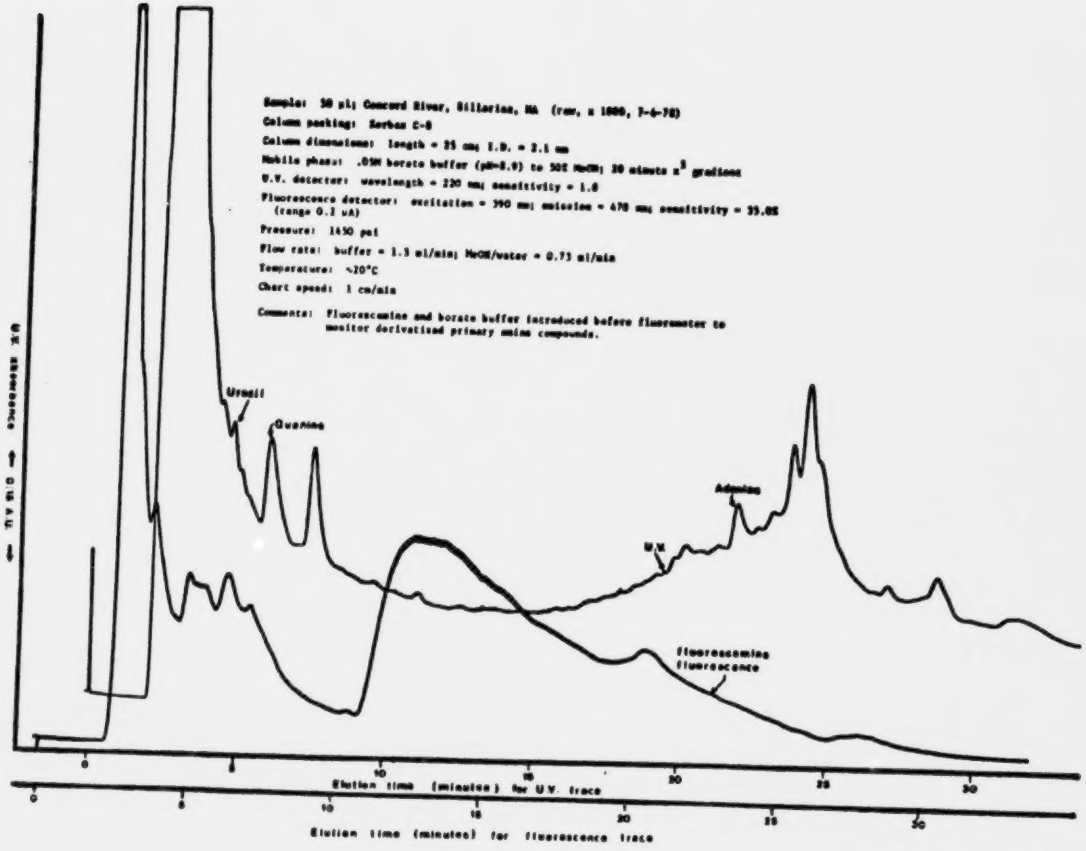


Figure 64. Chromatogram of raw Concord River sample (7/4/78), borate buffered eluant, with fluorescamine derivatized fluorescence.

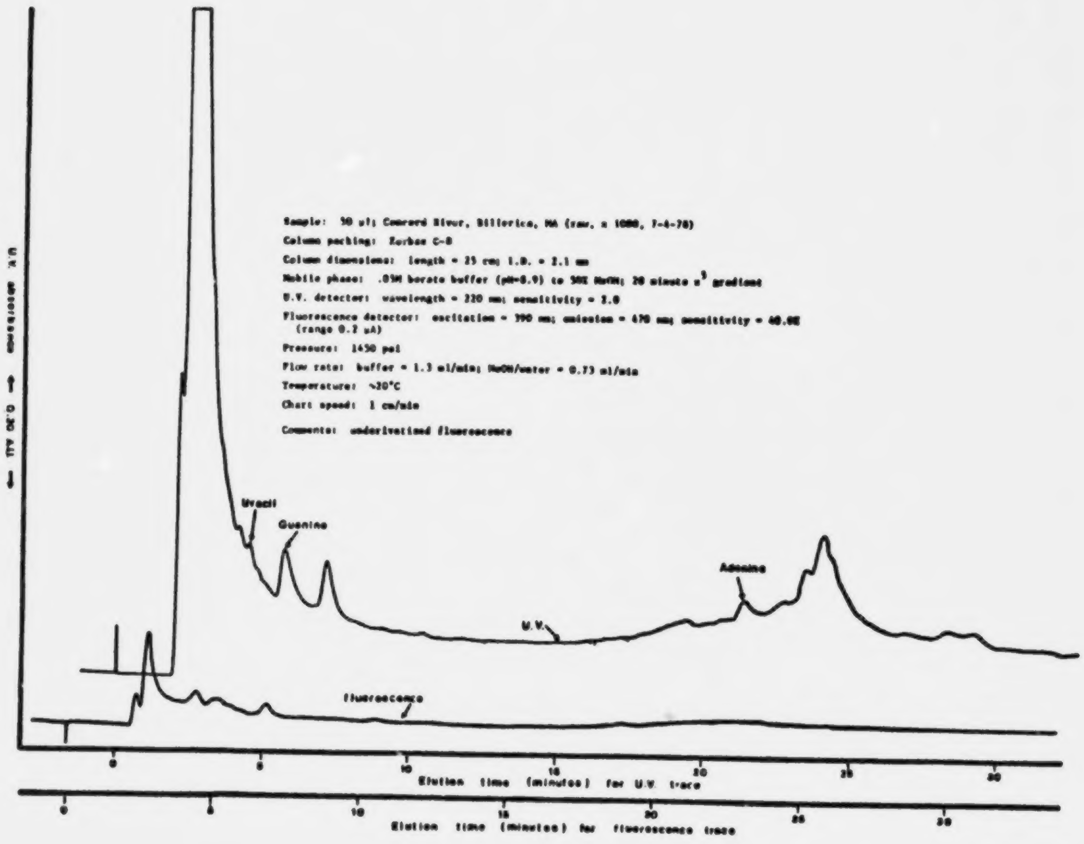
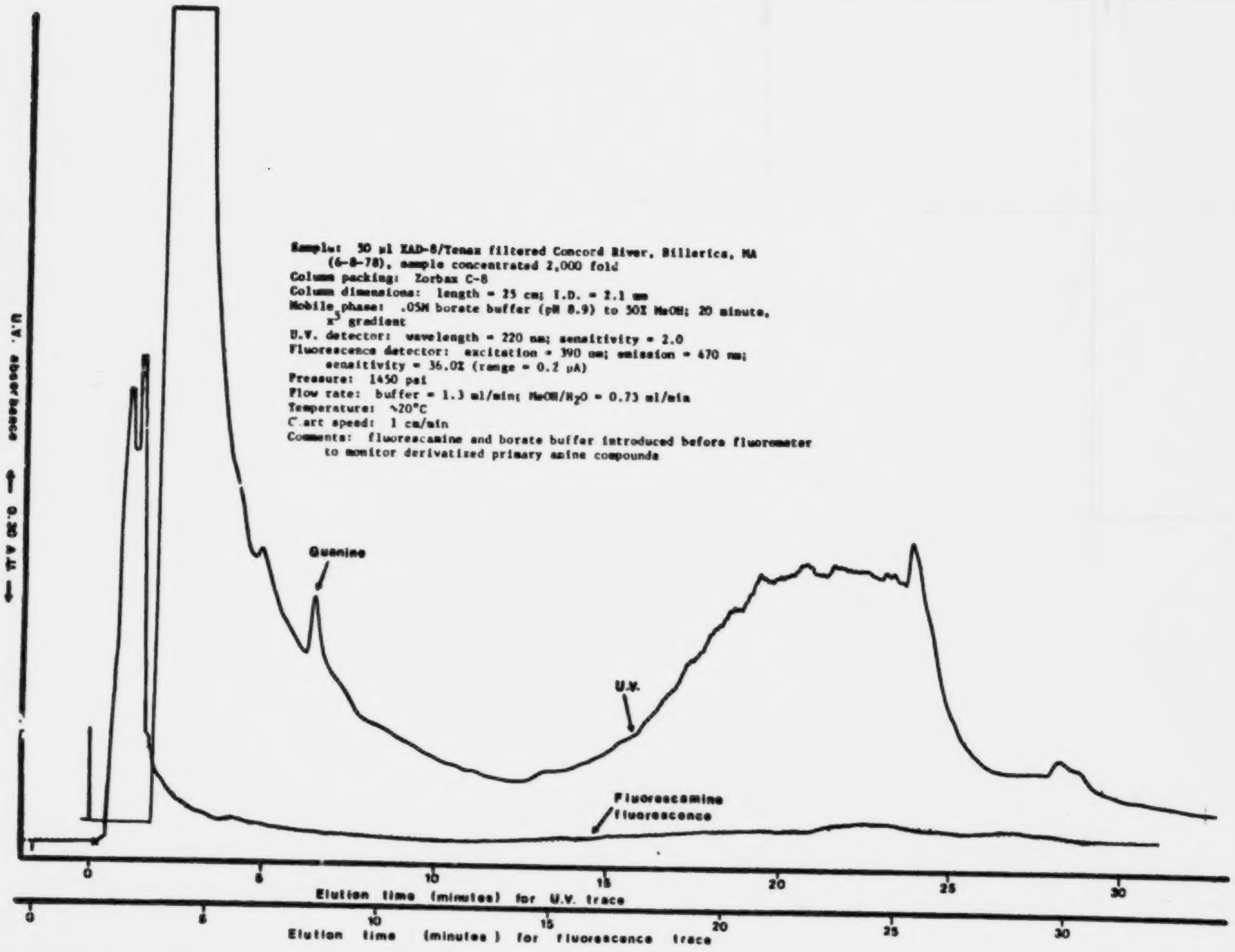
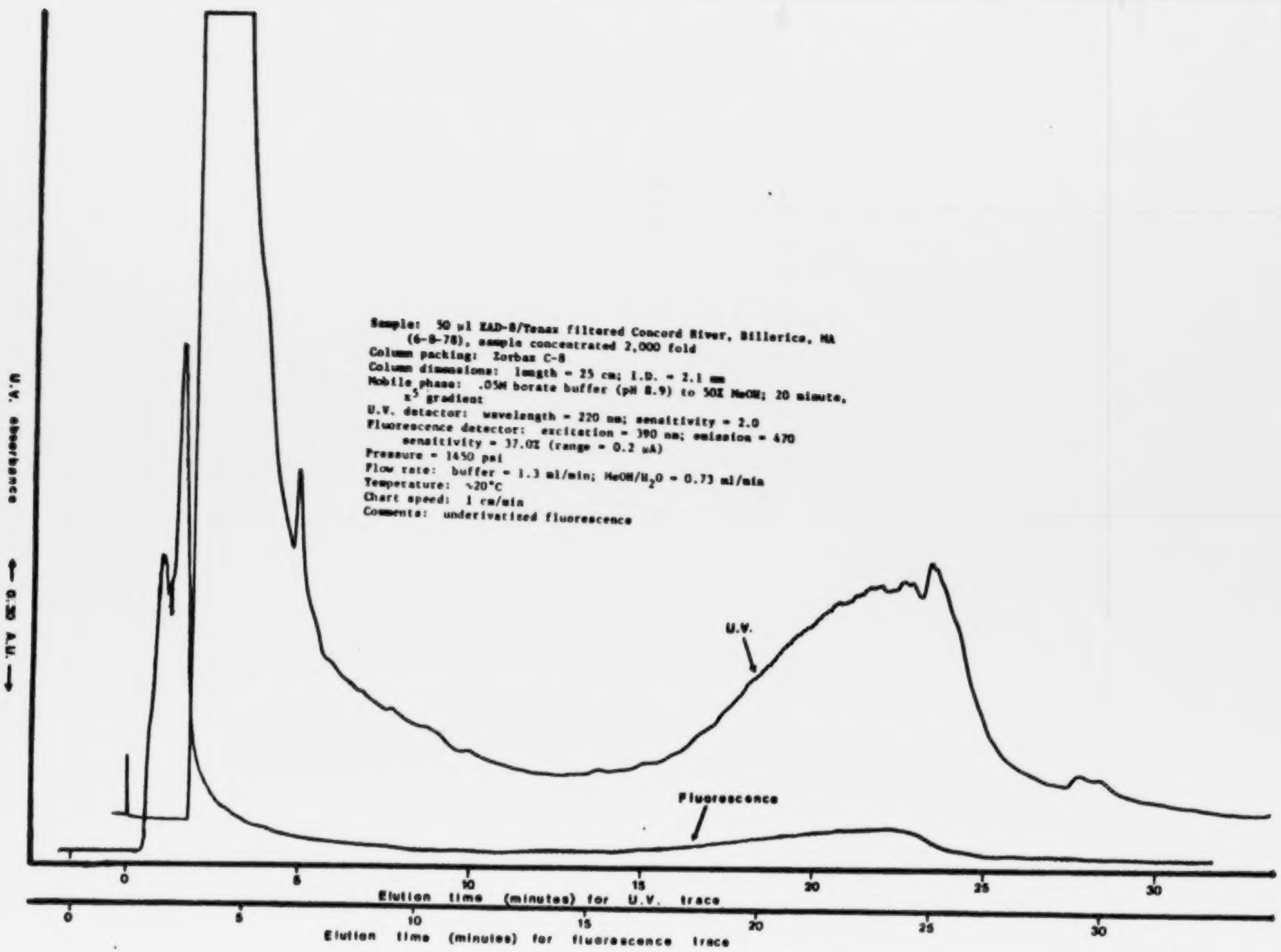


Figure 65. Chromatogram of raw Concord River sample (7/4/78), borate buffered eluant, with underivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered Concord River, Billerica, MA (6-8-78), sample concentrated 2,000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH 8.9) to 50% MeOH; 20 minute, x^3 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 36.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ∿20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds

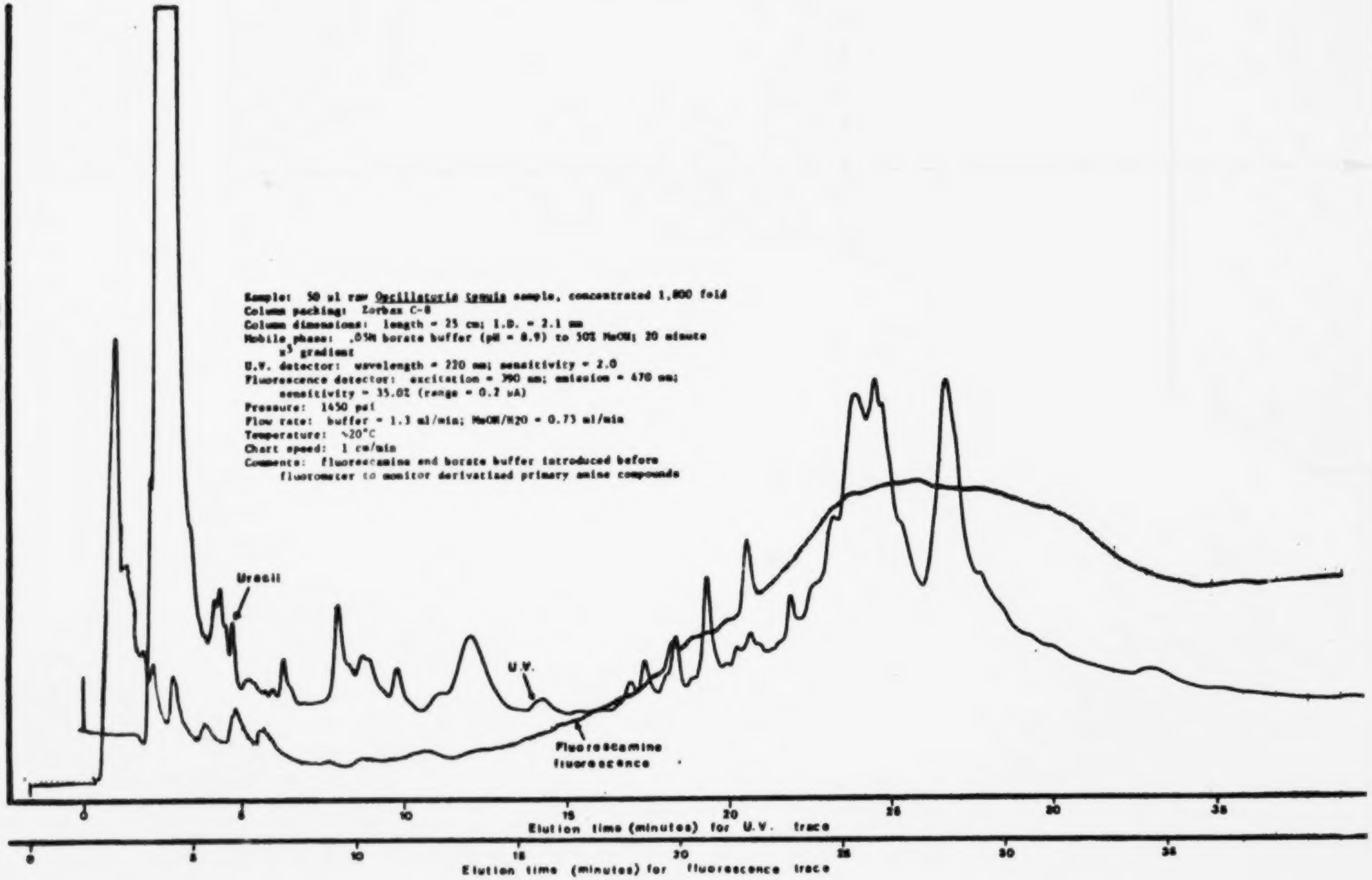
Figure 66. Chromatogram of resin-filtered Concord River sample (6/8/78), borate buffered eluant, with fluorescamine derivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered Concord River, Billerica, MA
(6-8-78), sample concentrated 2,000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH 8.9) to 50% MeOH; 20 minute, x^5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470
sensitivity = 37.0% (range = 0.2 µA)
Pressure = 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: underivatized fluorescence

U.V. absorbance
← 0.30 A.U. →
U.V.
Fluorescence
0
5
10
15
20
25
30
Elution time (minutes) for U.V. trace
0
5
10
15
20
25
30
Elution time (minutes) for fluorescence trace

Figure 67. Chromatogram of resin-filtered Concord River sample (6/9/78), borate buffered eluant, with underivatized fluorescence.



Sample: 50 µl raw Oscillatoria tenuis sample, concentrated 1,800 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute x^3 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 35.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds

Figure 68. Chromatogram of raw filtrate from a culture of *Oscillatoria tenuis*, borate buffered eluant, with fluorescamine derivatized fluorescence.

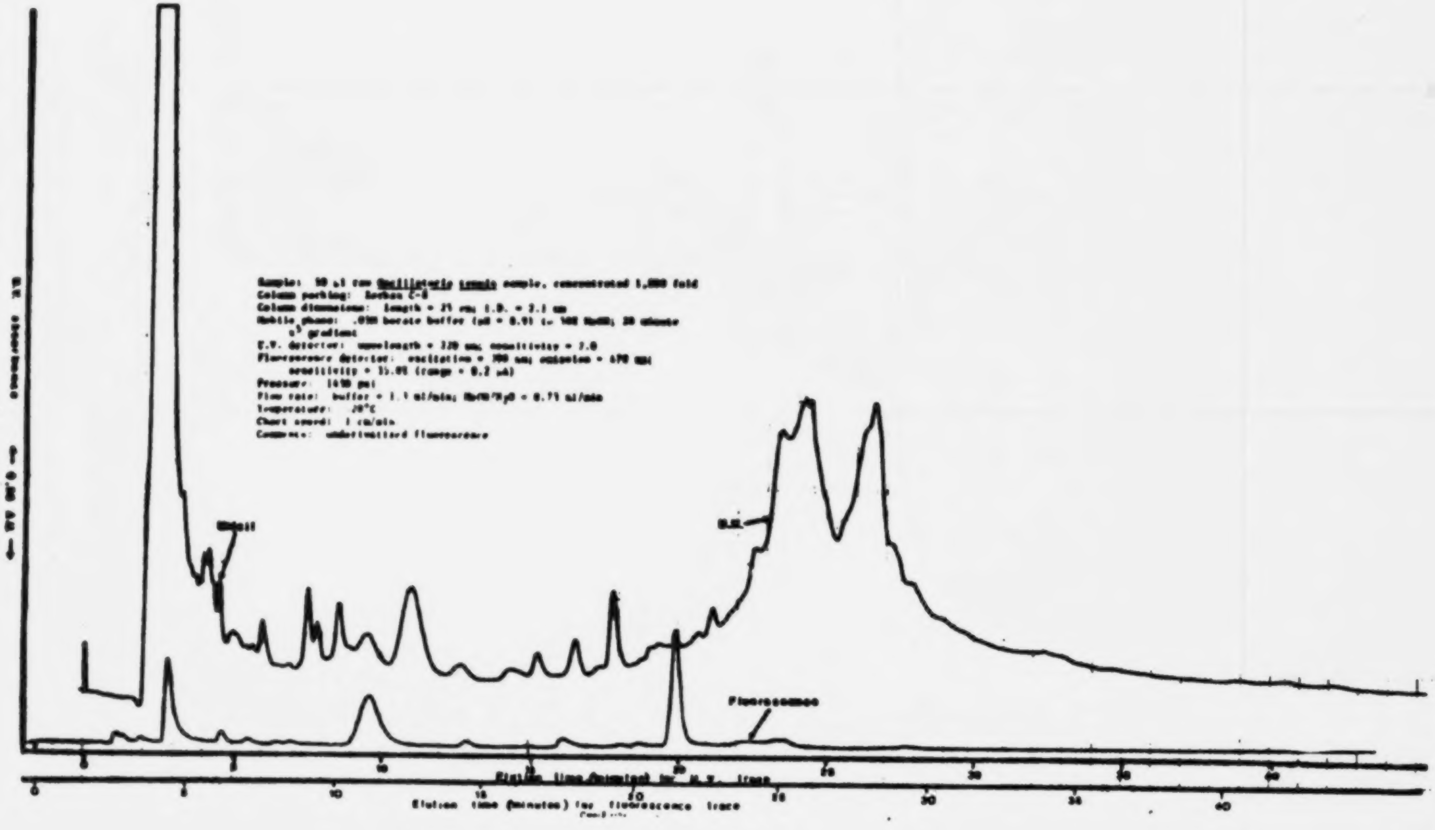
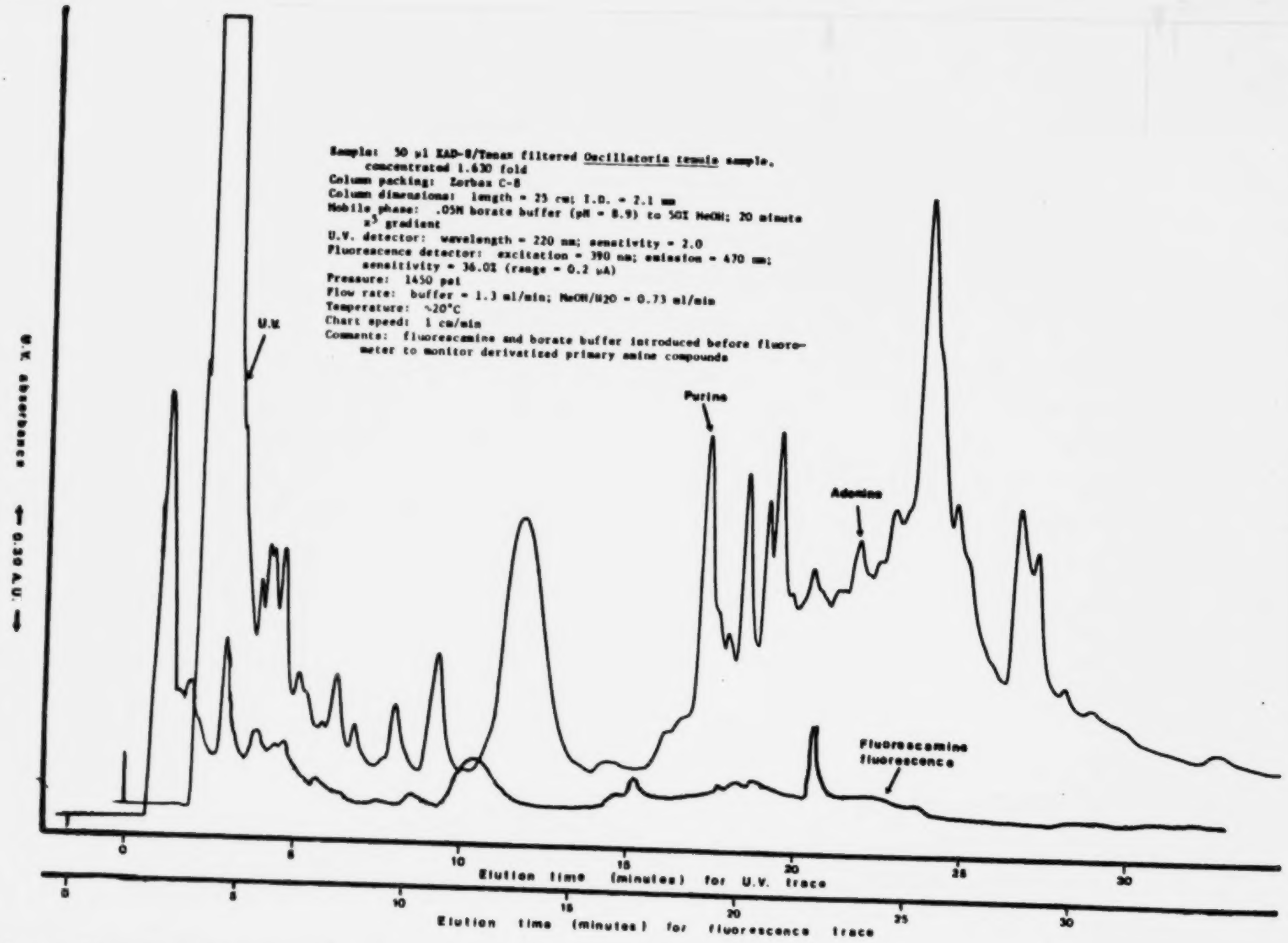


Figure 69. Chromatogram of raw filtrate from a culture of *Oscillatoria tenuis*, borate buffered eluant, with underivatized fluorescence.



Sample: 50 μl XAD-8/Tenax filtered Oscillatoria tenuis sample, concentrated 1.630 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute x^3 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 36.0% (range = 0.2 μA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds

Figure 70. Chromatogram of resin-filtered filtrate from a culture of *Oscillatoria tenuis*, borate buffered eluant, with fluorescamine derivatized fluorescence.

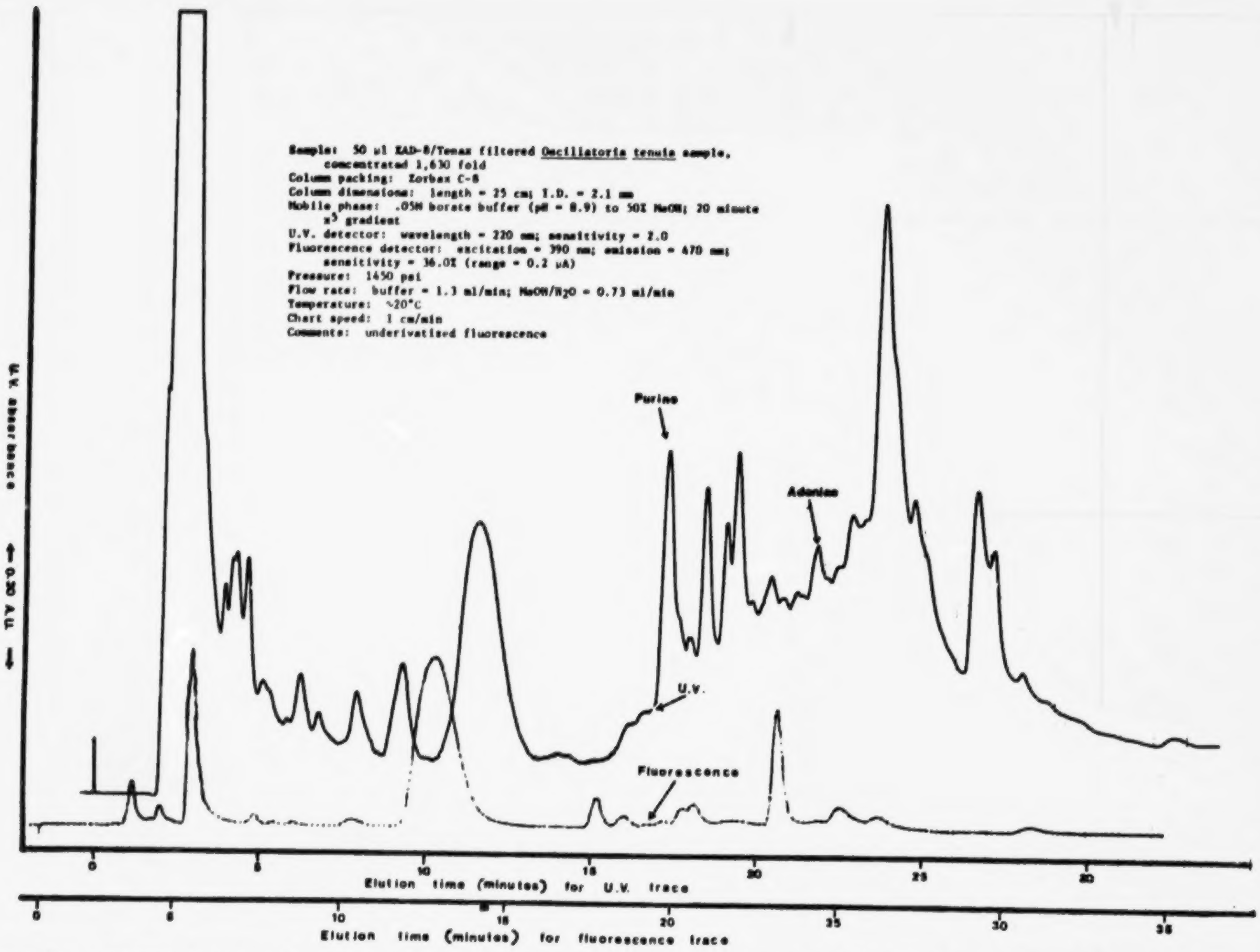


Figure 71. Chromatogram of resin-filtered filtrate from a culture of *Oscillatoria tenuis*, borate buffered eluant, with underivatized fluorescence.

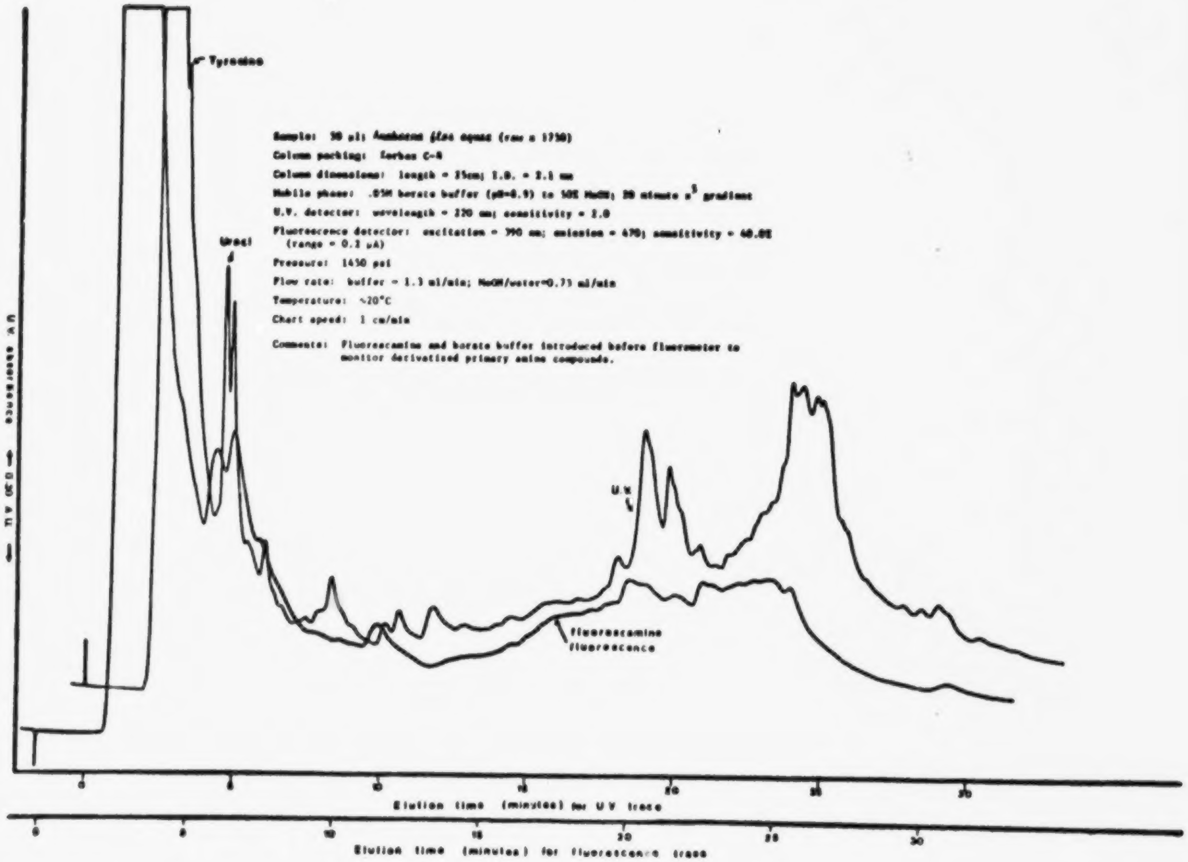


Figure 72. Chromatogram of raw filtrate from a culture of *Anabaena flos aquae*, borate buffered eluant, with fluorescamine derivatized fluorescence.

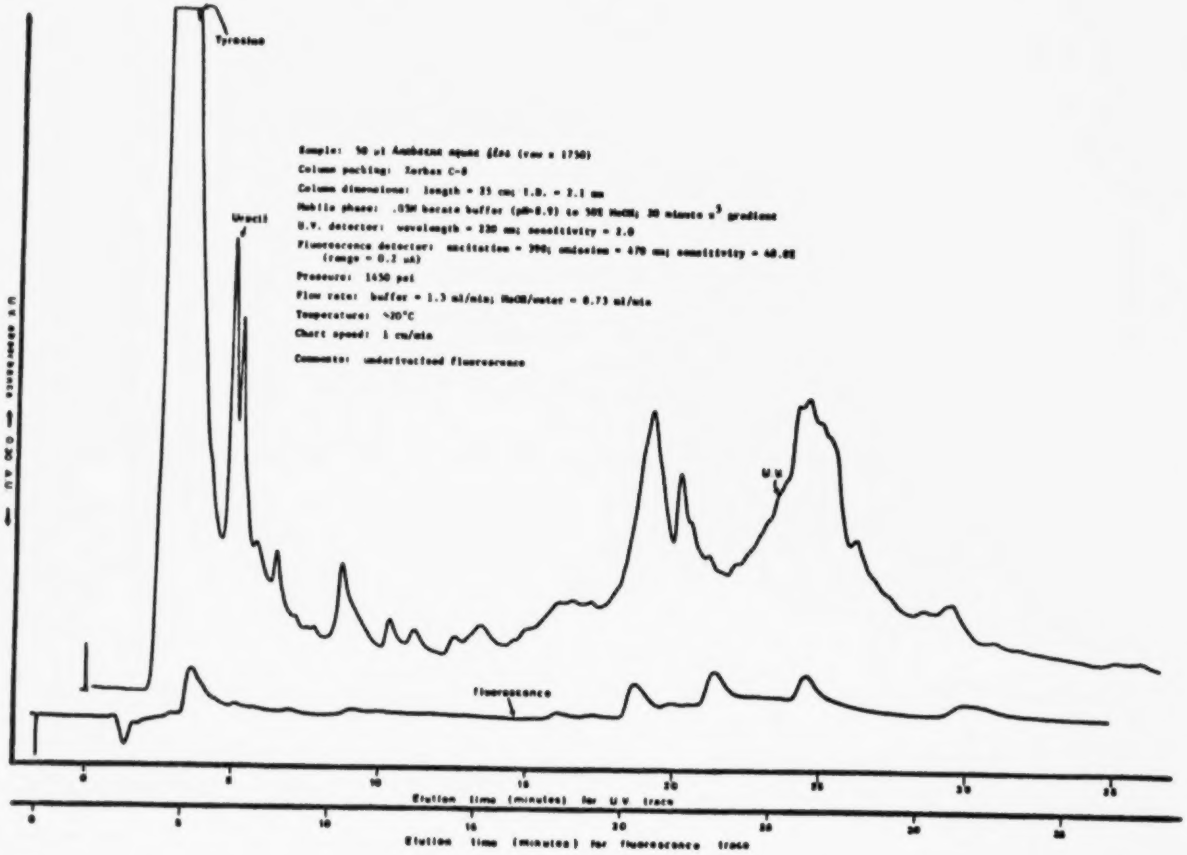


Figure 73. Chromatogram of raw filtrate from a culture of *Anabaena flos aquae*, borate buffered eluant, with underivatized fluorescence.

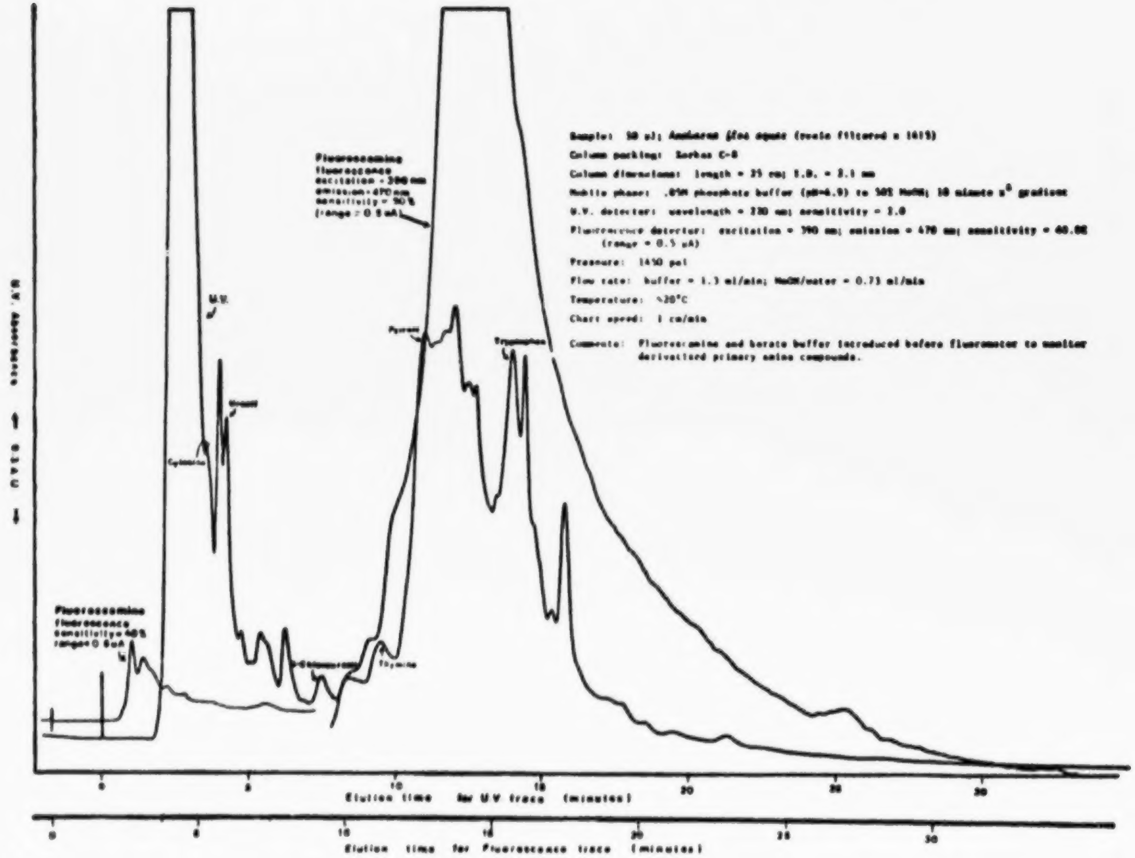


Figure 74. Chromatogram of resin-filtered filtrate from a culture of *Anabaena flos aquae*, phosphate buffered eluant, fluorescamine derivatized fluorescence.

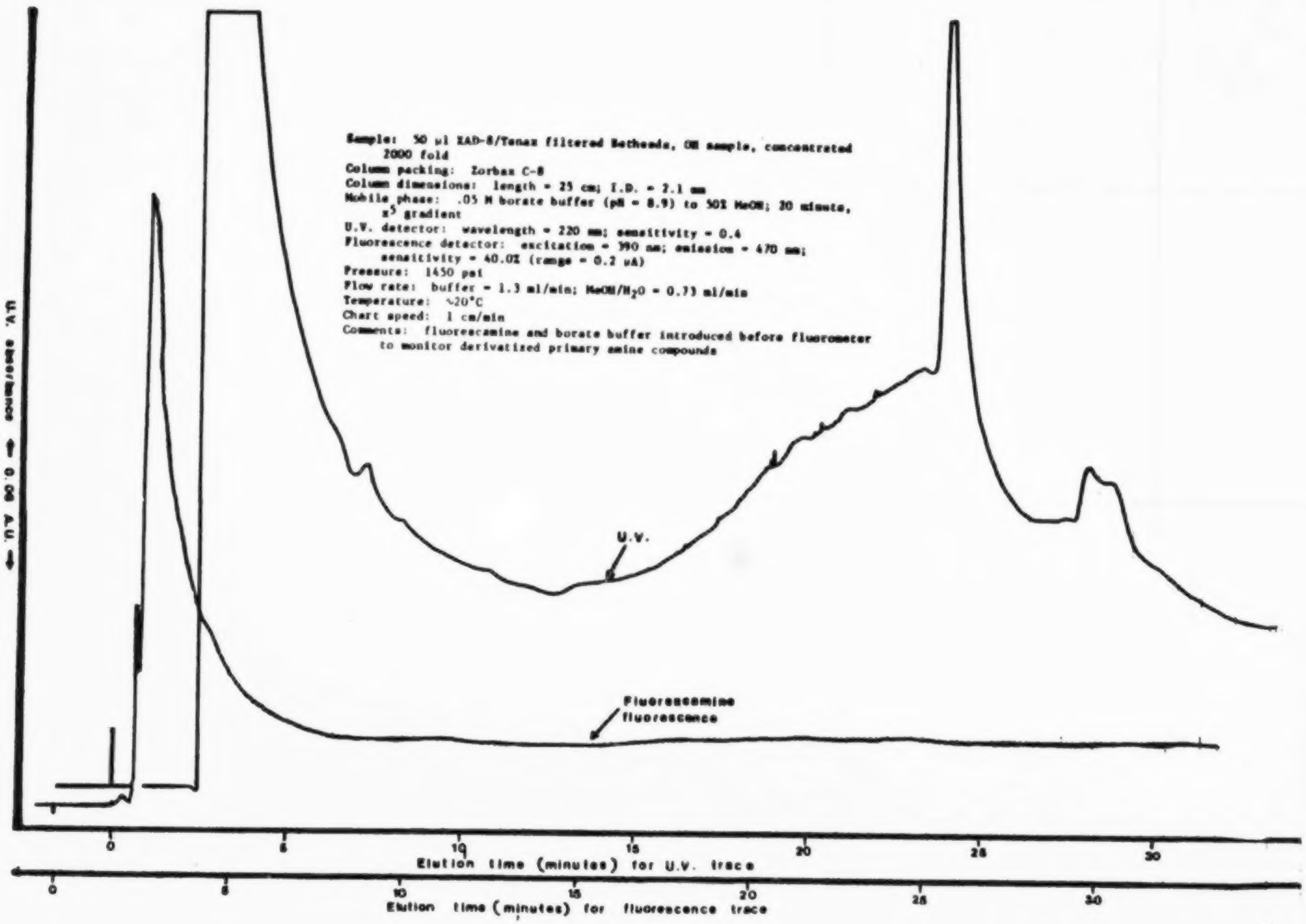
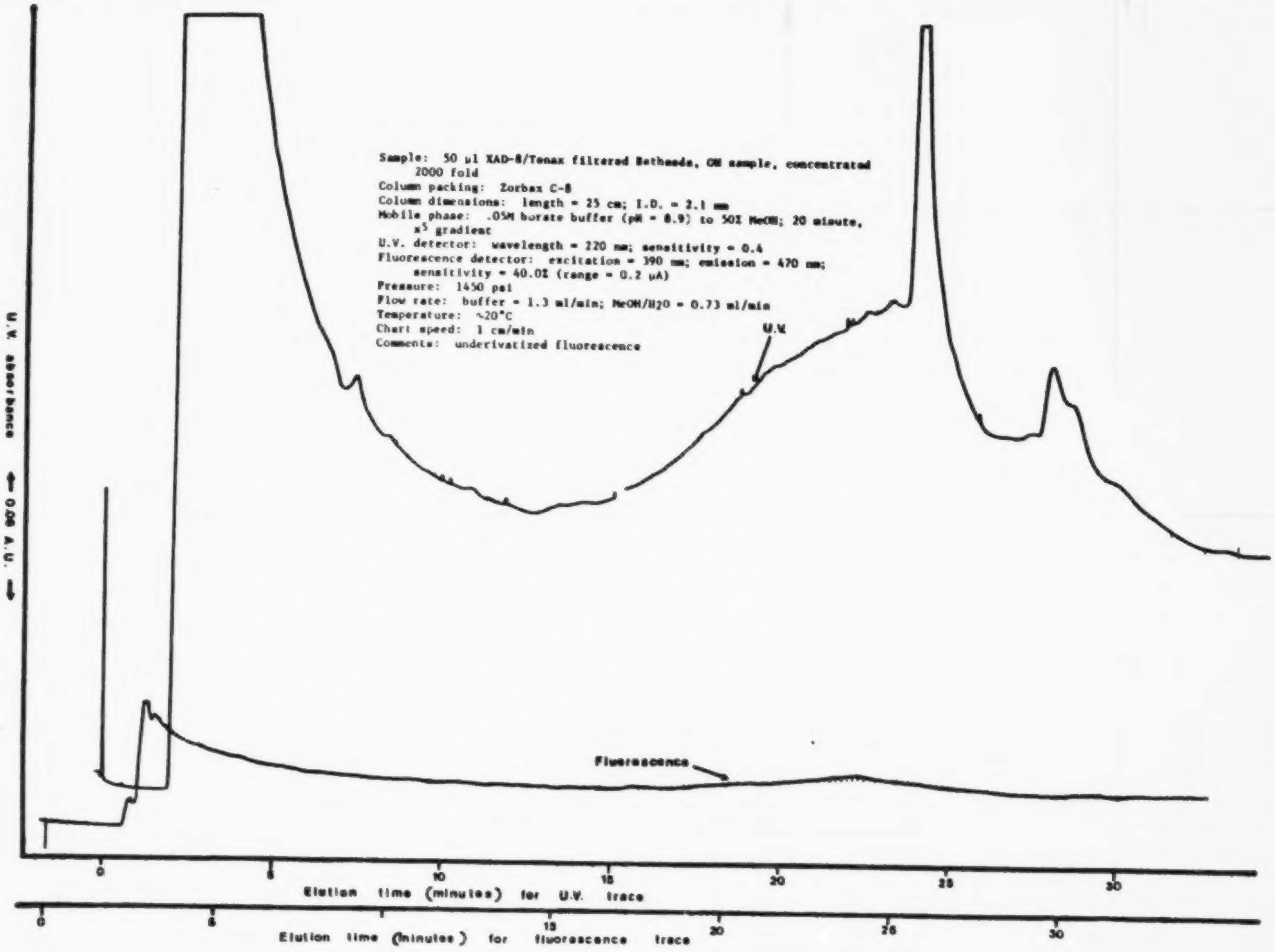


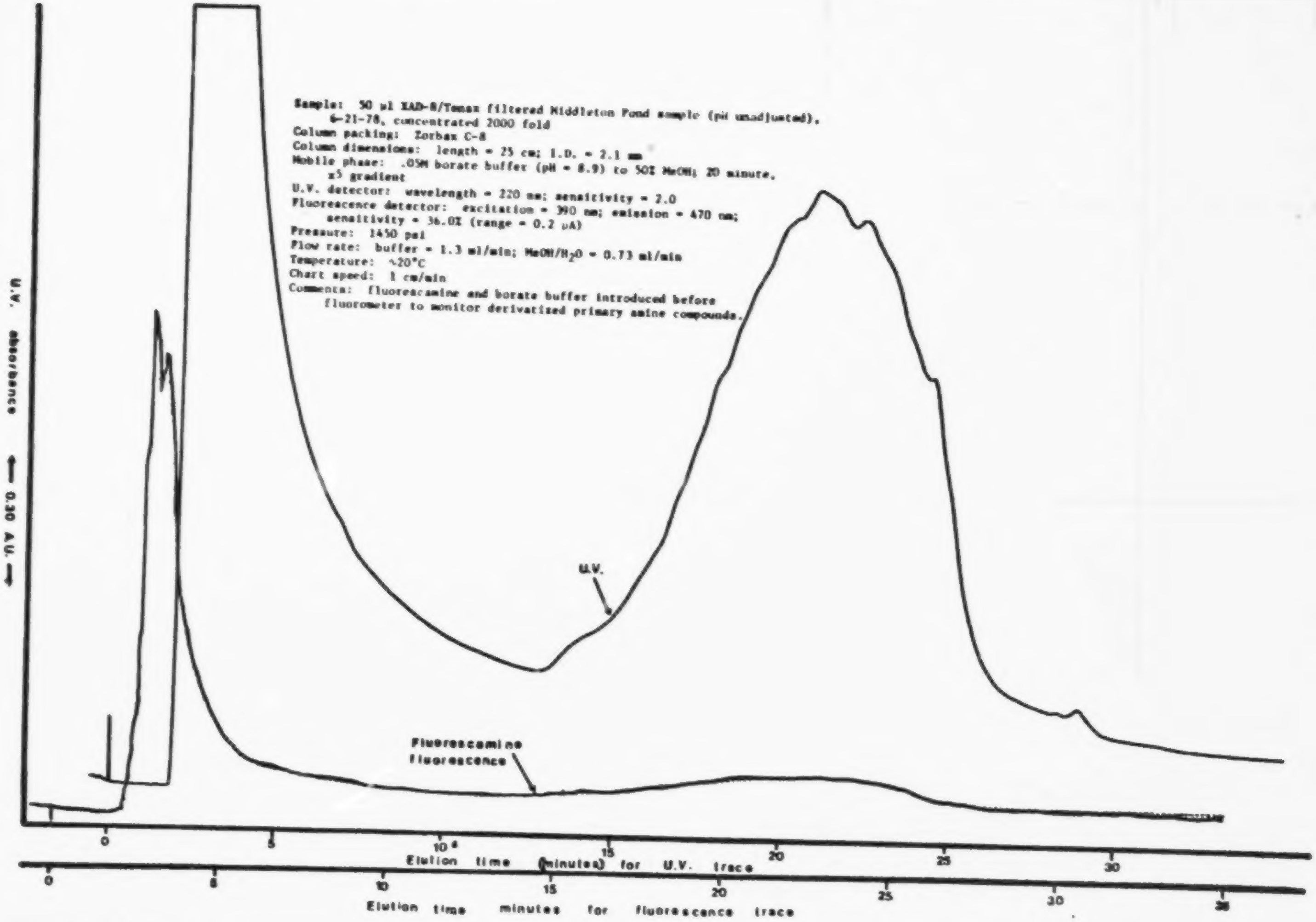
Figure 75. Chromatogram of resin-filtered surface impoundment sample, Bethesda, Ohio, borate buffered eluant, with fluorescamine fluorescence.



Sample: 50 µl XAD-8/Tenax filtered Bethesda, OH sample, concentrated 2000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute, x^5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 0.4
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 40.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: underivatized fluorescence

U.V.
Fluorescence
U.V. absorbance ← 0.08 A.U. →
0
5
10
15
20
25
30
Elution time (minutes) for U.V. trace
0
5
15
20
25
30
Elution time (minutes) for fluorescence trace

Figure 76. Chromatogram of raw surface impoundment sample, Bethesda, Ohio, borate buffered eluant, with underivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered Middleton Pond sample (pH unadjusted), 6-21-78, concentrated 2000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute, x5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 36.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds.

U.V. absorbance ⟵ 0.30 A.U. ⟶
U.V.
Fluorescamine fluorescence
0
5
10
15
20
25
30
Elution time (minutes) for U.V. trace
0
5
10
15
20
25
30
35
Elution time minutes for fluorescence trace

Figure 77. Chromatogram of resin-filtered (pH unadjusted) Middleton Pond sample (6/21/78), borate buffered eluant, with fluorescamine derivatized fluorescence.

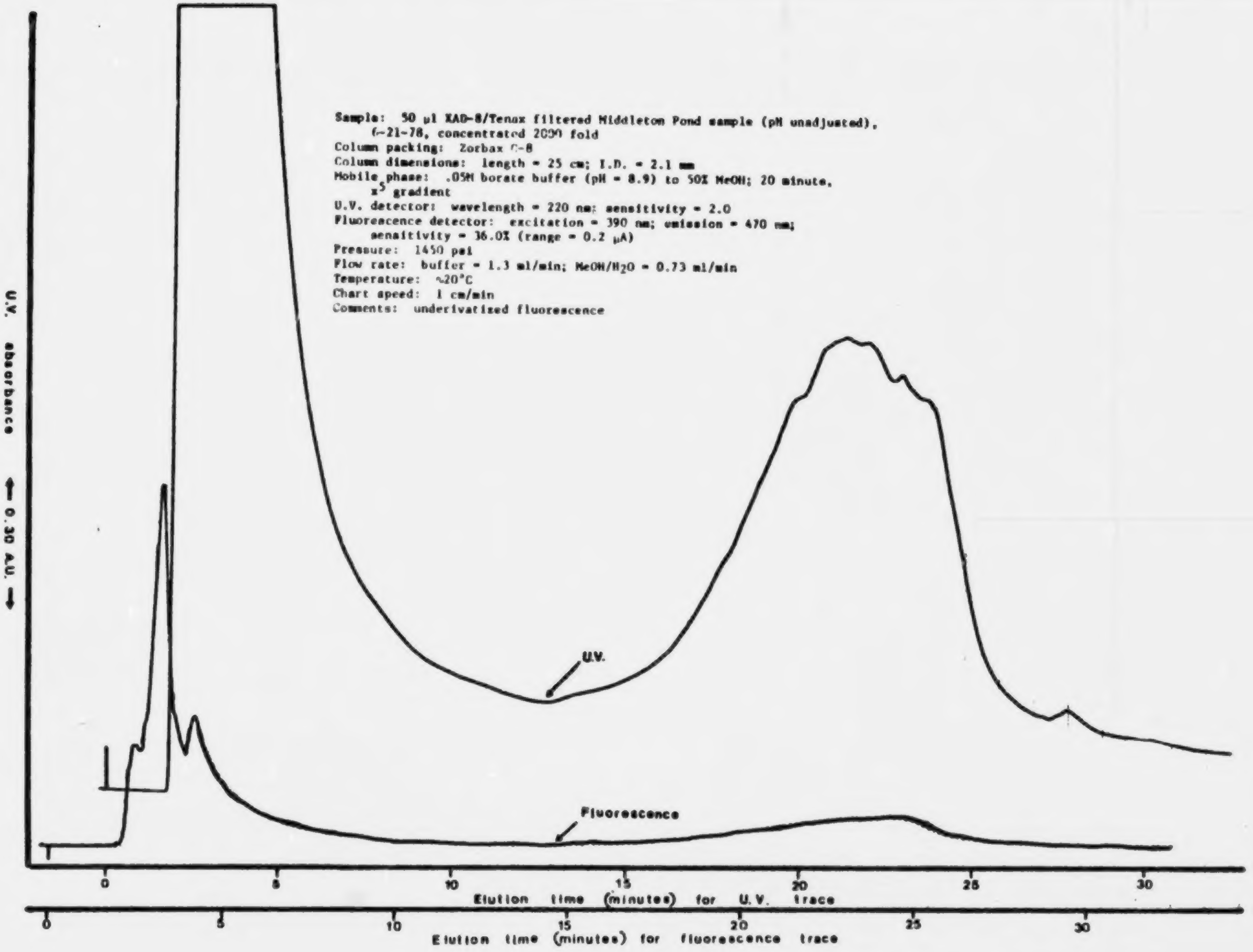
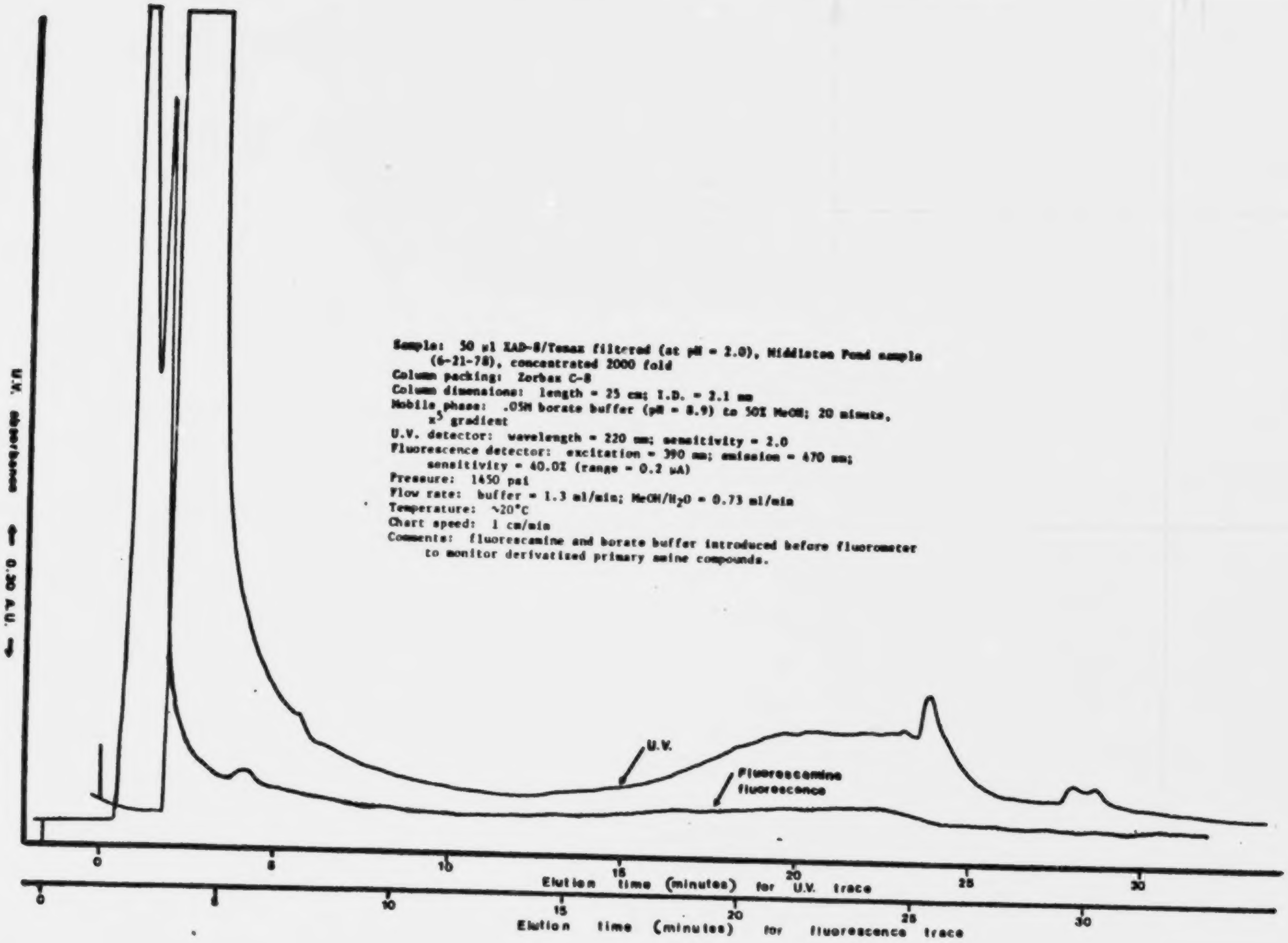
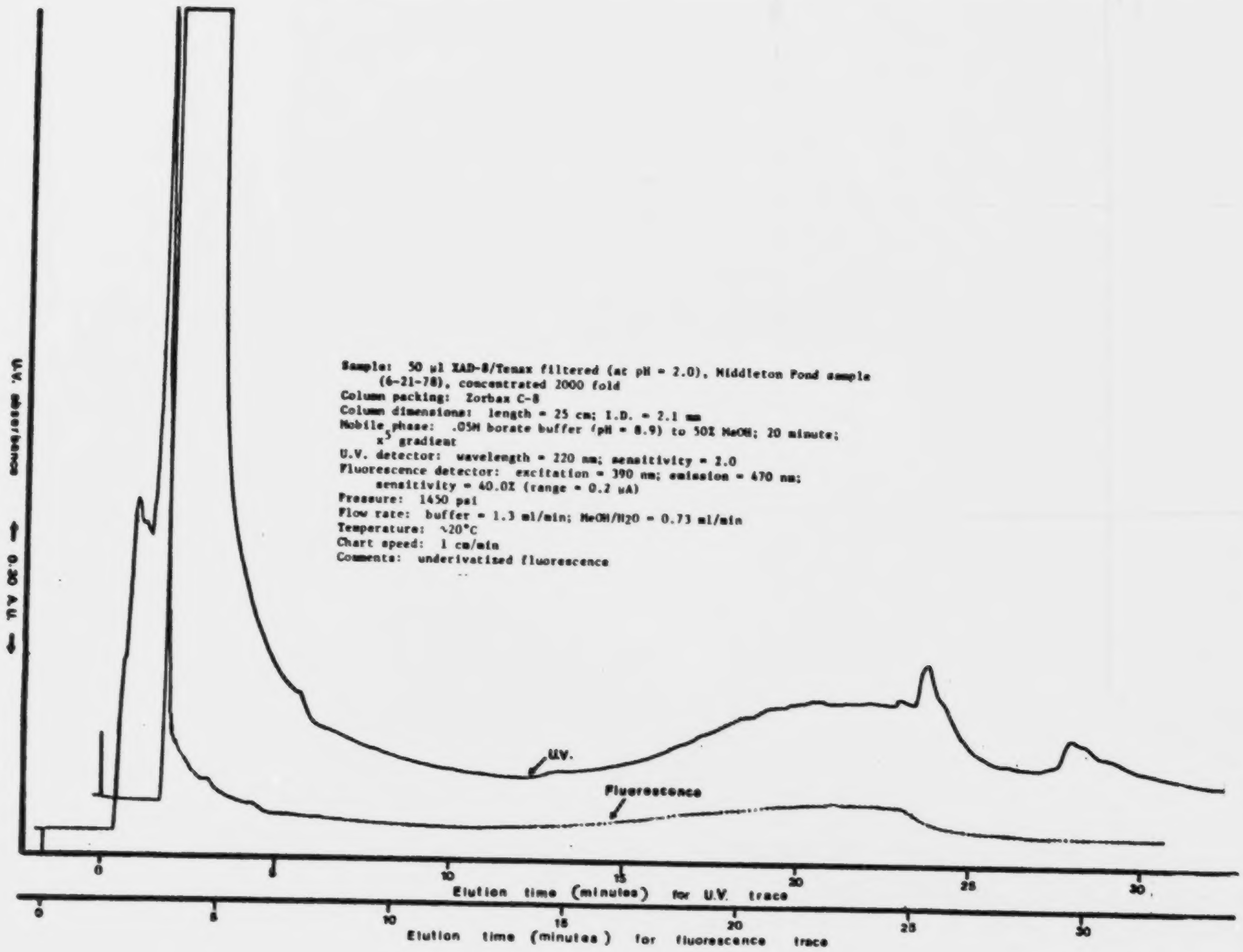


Figure 78. Chromatogram of resin-filtered (pH unadjusted) Middleton Pond sample (6/21/78), borate buffered eluant, with underivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered (at pH = 2.0), Middleton Pond sample (6-21-78), concentrated 2000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute, x^5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 40.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H_2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds.

Figure 79. Chromatogram of resin-filtered (at pH = 2), Middleton Pond sample (6/21/78), borate buffered eluant, with fluorescamine derivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered (at pH = 2.0), Middleton Pond sample (6-21-78), concentrated 2000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute; x^5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 40.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: underivatized fluorescence

Figure 80. Chromatogram of resin filtered (at pH = 2), Middleton Pond sample (6/21/78), borate buffered eluant, with underivatized fluorescence.

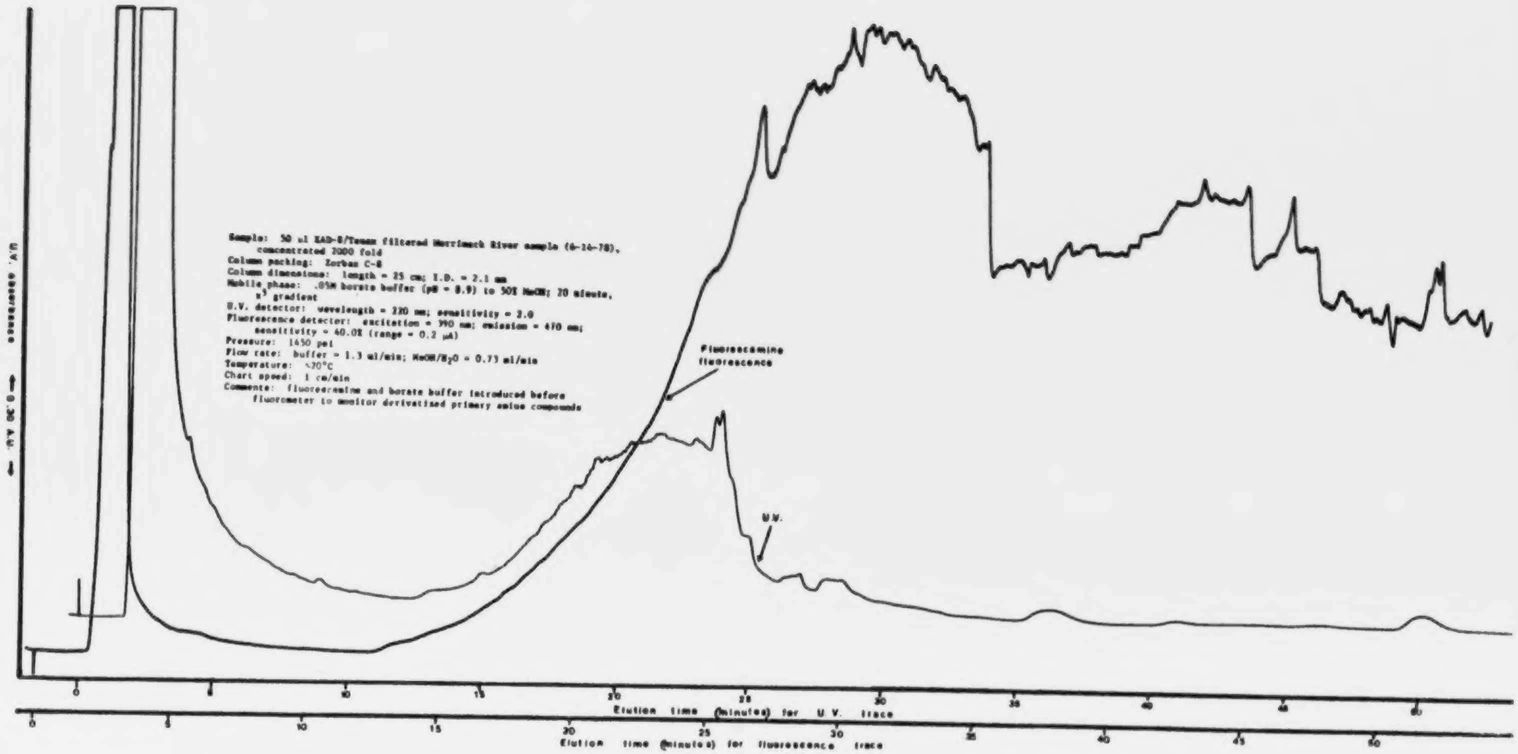
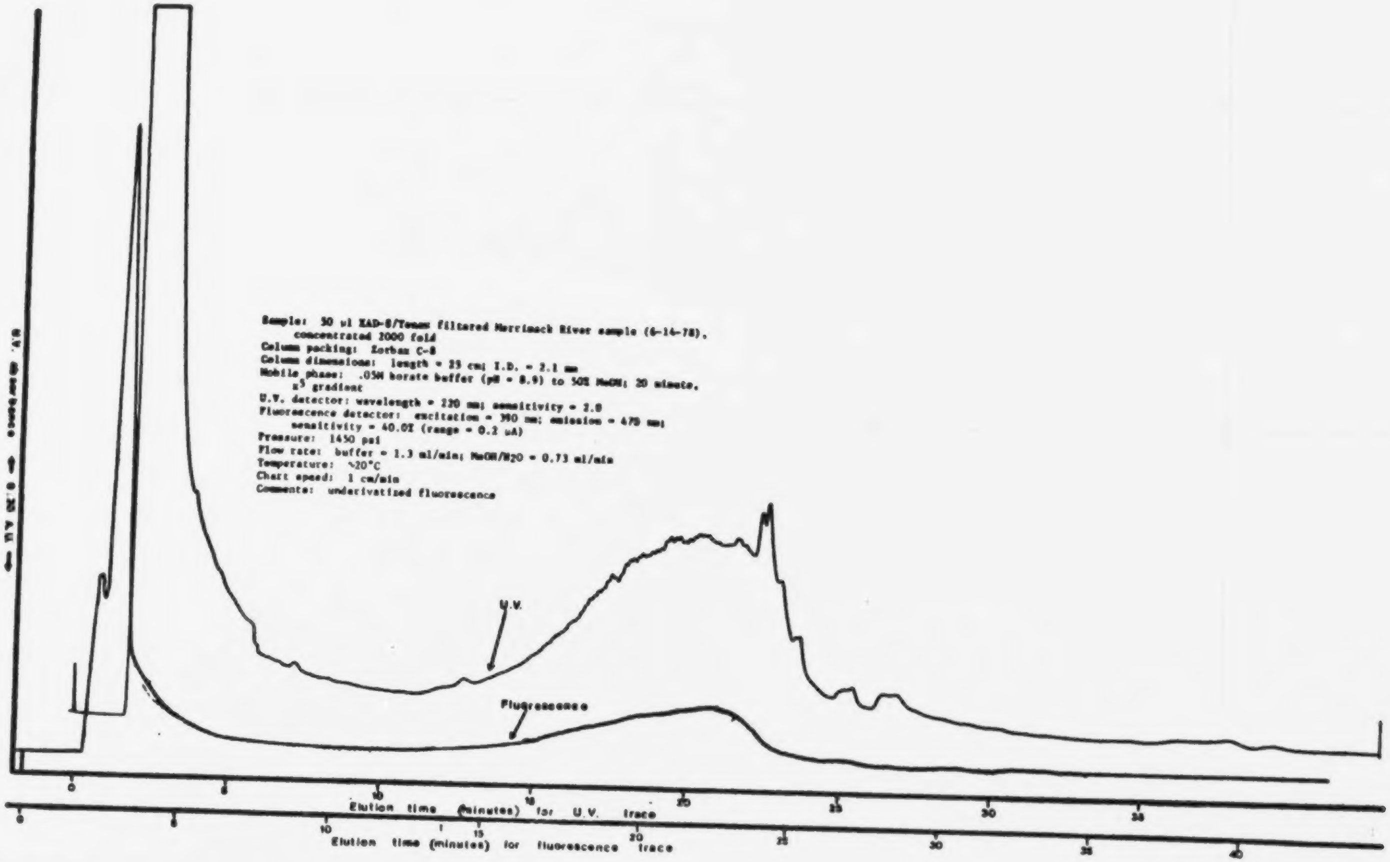


Figure 81. Chromatogram of resin-filtered Merrimack River sample (6/14/78), borate buffered eluant, with fluorescamine derivatized fluorescence.



Sample: 50 µl XAD-8/Tenax filtered Merrimack River sample (6-14-78), concentrated 2000 fold
Column packing: Zorbax C-8
Column dimensions: length = 25 cm; I.D. = 2.1 mm
Mobile phase: .05M borate buffer (pH = 8.9) to 50% MeOH; 20 minute, x^5 gradient
U.V. detector: wavelength = 220 nm; sensitivity = 2.0
Fluorescence detector: excitation = 390 nm; emission = 470 nm; sensitivity = 40.0% (range = 0.2 µA)
Pressure: 1450 psi
Flow rate: buffer = 1.3 ml/min; MeOH/H2O = 0.73 ml/min
Temperature: ~20°C
Chart speed: 1 cm/min
Comments: underivatized fluorescence

Figure 82. Chromatogram of resin-filtered Merrimack River sample (6/14/78), borate buffered eluant, with underivatized fluorescence.

Identification of unknown resolved chromatographic peaks was achieved by comparison of retention position and U.V. data with those of reference compounds. U.V. data were obtained by stopped-flow spectral scanning of individual chromatographic peaks. It was found, however, that the retention positions for some compounds were effected by the stopped-flow analytical method. For example, after scanning cytosine using the stopped flow technique, it was found that thymine eluted only 2.5 minutes after resumption of mobile phase flow through the Zorbax C-8 column 'a' using the phosphate to methanol gradient. With continuous eluant flow, thymine eluted 4.5 minutes after cytosine. The shorter elution time of thymine, observed in the separation where the mobile phase had been temporarily stopped, indicated that this compound probably continued to diffuse through the column while the eluant was static. Pyrrole, on the other hand, was eluted the same amount of time after thymine, with or without stopping of the mobile phase during the chromatographic run. Positions of unknown chromatographic peaks could therefore be unambiguously related to those of reference materials only in that portion of the chromatogram obtained prior to and including the first stopped-flow spectral scan of an eluting material. Stopped-flow spectral scanning of unknown compounds eluting at positions of reference materials was therefore done only one time for each chromatographic run.

The principal information used in comparing U.V. spectra of unknown materials with those of reference compounds was the positions of maxima and minima U.V. absorbance, and ratios of maximum to minimum U.V. absorbance and absorbance at 220 nm to 233 nm. U.V. spectra of materials isolated from concentrated field samples are shown in Figures 83 to 109. A summary of the information taken from these spectra for use in the identification of these materials is shown in Table 29.

The sample taken at Middleton Pond, Danvers, MA (7/11/78) was analyzed using both the borate and phosphate buffer to methanol gradients on the first Zorbax C-8 column (column 'a'). The probability that the chromatographic peaks were correctly identified in this sample increased because of the corroborative retention data and U.V. spectra obtained using the two different operating conditions. Unfortunately, loss in retentivity on replacement Zorbax C-8 columns using phosphate buffer as the mobile phase, precluded the use of this eluant in obtaining corroborative information on other samples.

Absorbance ratio values of sample to reference compound equal to 1 represented a perfect correlation of the unknown compound to the reference material. Values above or below 1 were attributable to increased or decreased absorbance in the unknown material arising from the presence of coeluting compounds.

Table 30 lists the samples which did not result in identifiable chromatographic peaks, In some cases only one large unresolved group of compounds, rapidly eluting from the column, was observed. Other chromatograms contained distinct peaks which did not coincide with the retention positions of any of the reference nitrogenous compounds (Figures 75 to 82).

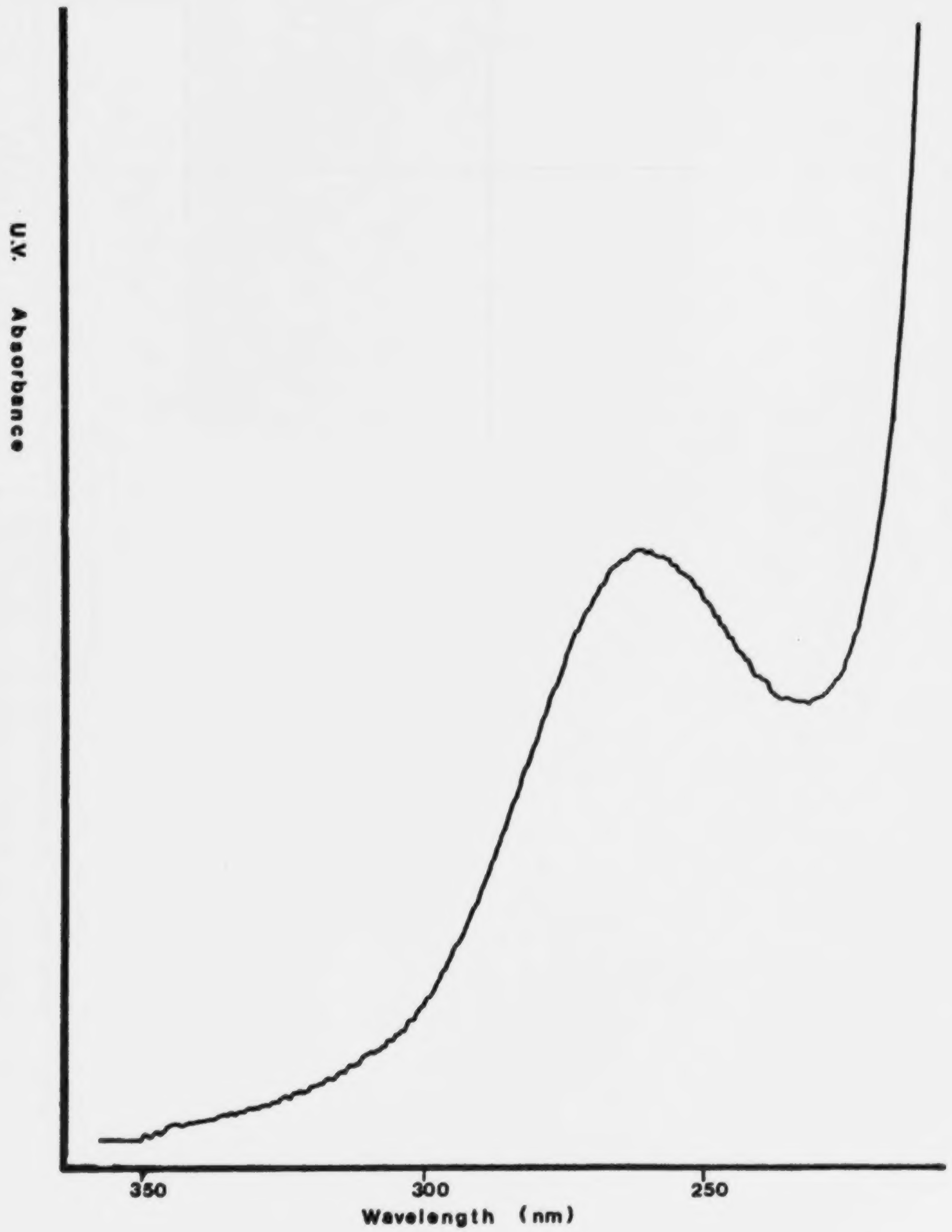


Figure 83. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Phosphate buffered eluant, suspected compound: uracil.

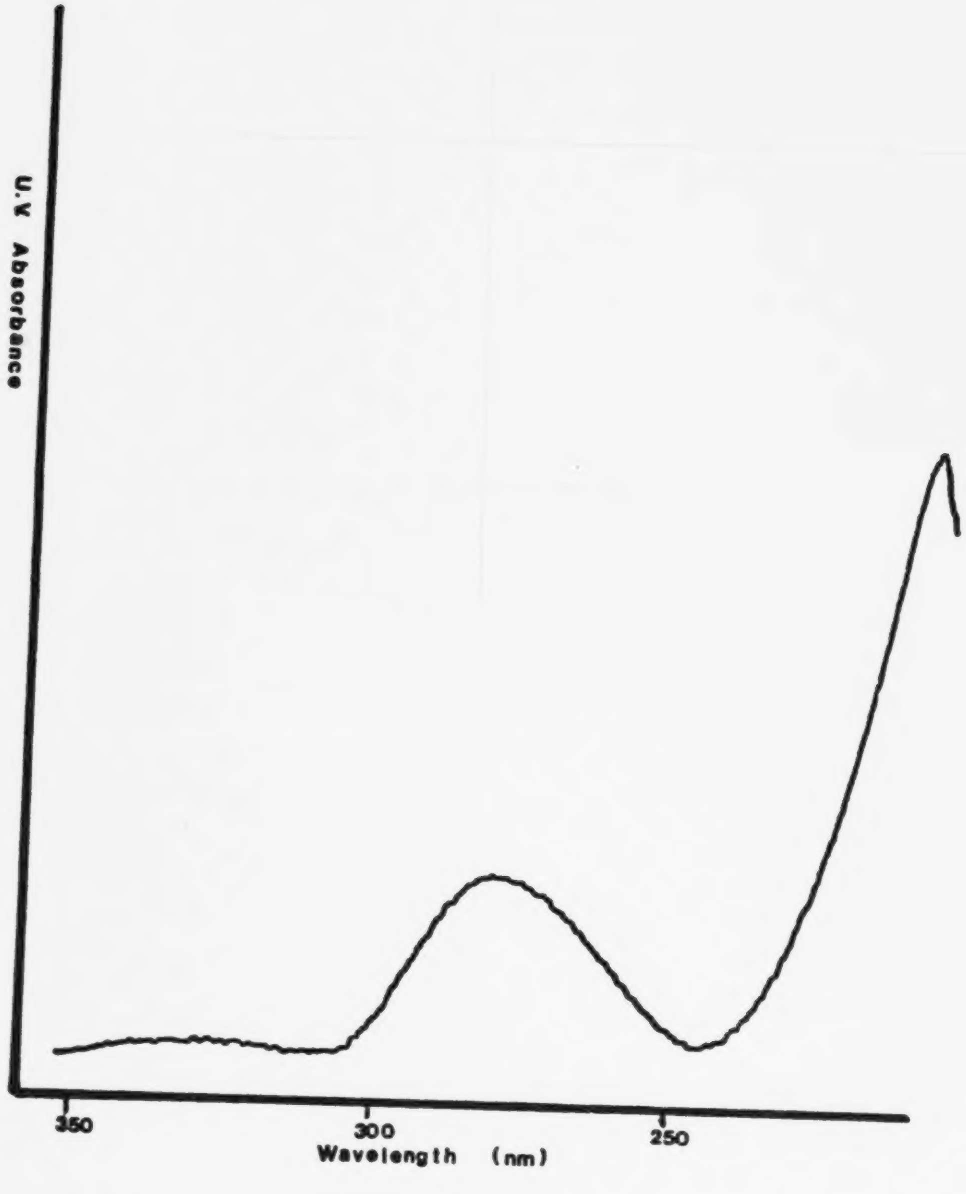


Figure 84. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Phosphate buffered eluant, suspected compound: 5-chlorouracil.

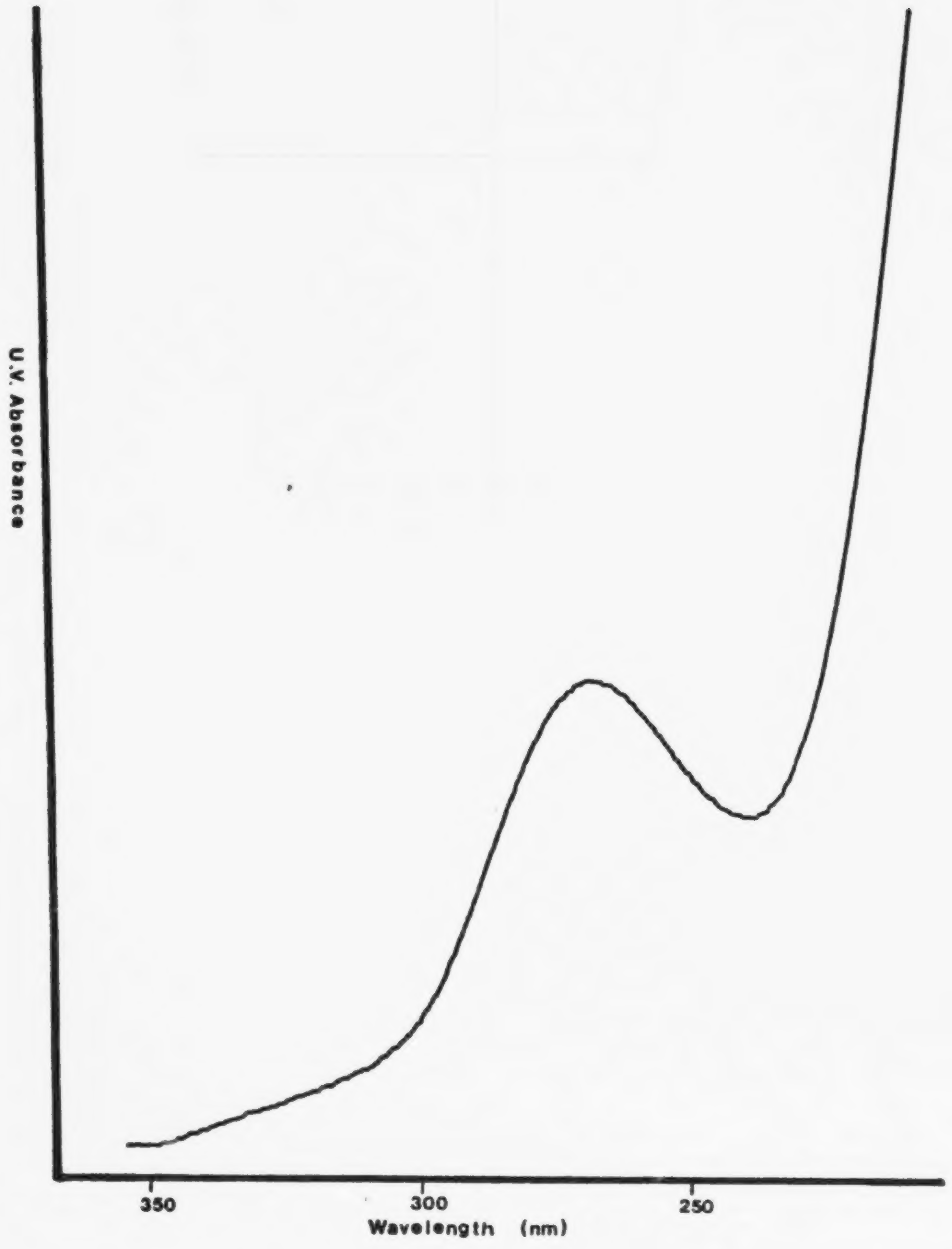


Figure 85. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Phosphate buffered eluant, suspected compound: thymine.

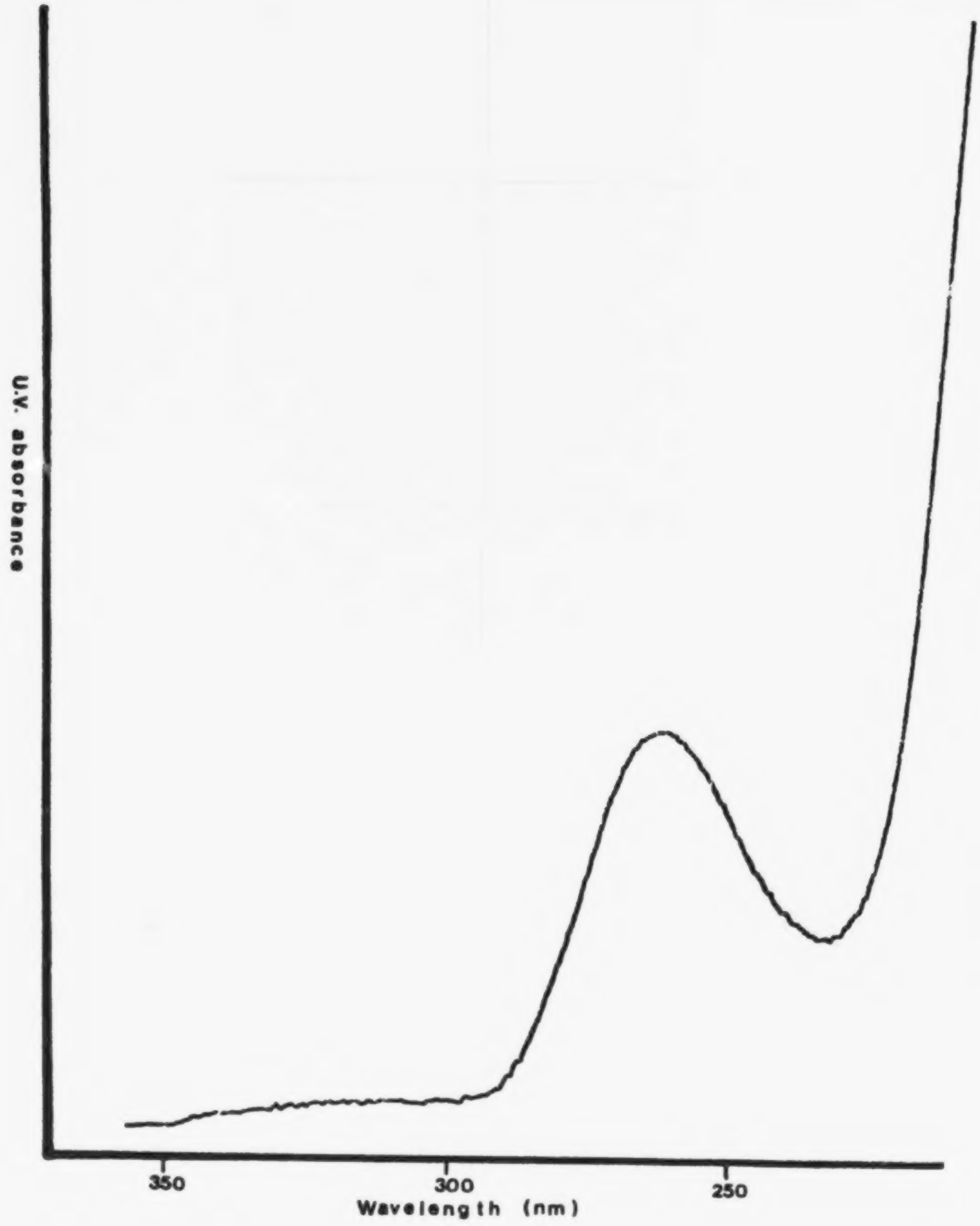


Figure 86. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Phosphate buffered eluant, suspected compound: adenine.

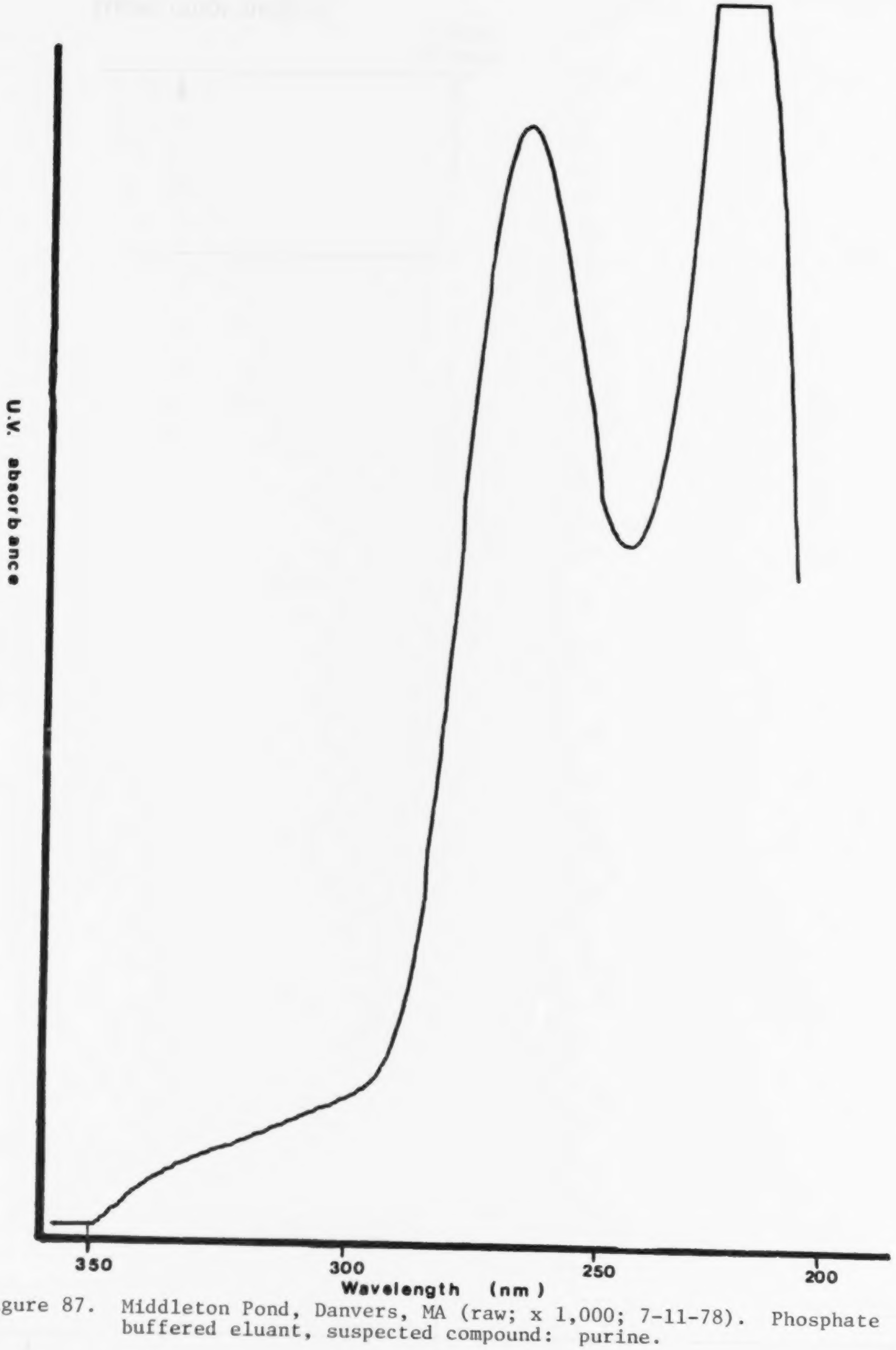


Figure 87. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Phosphate buffered eluant, suspected compound: purine.

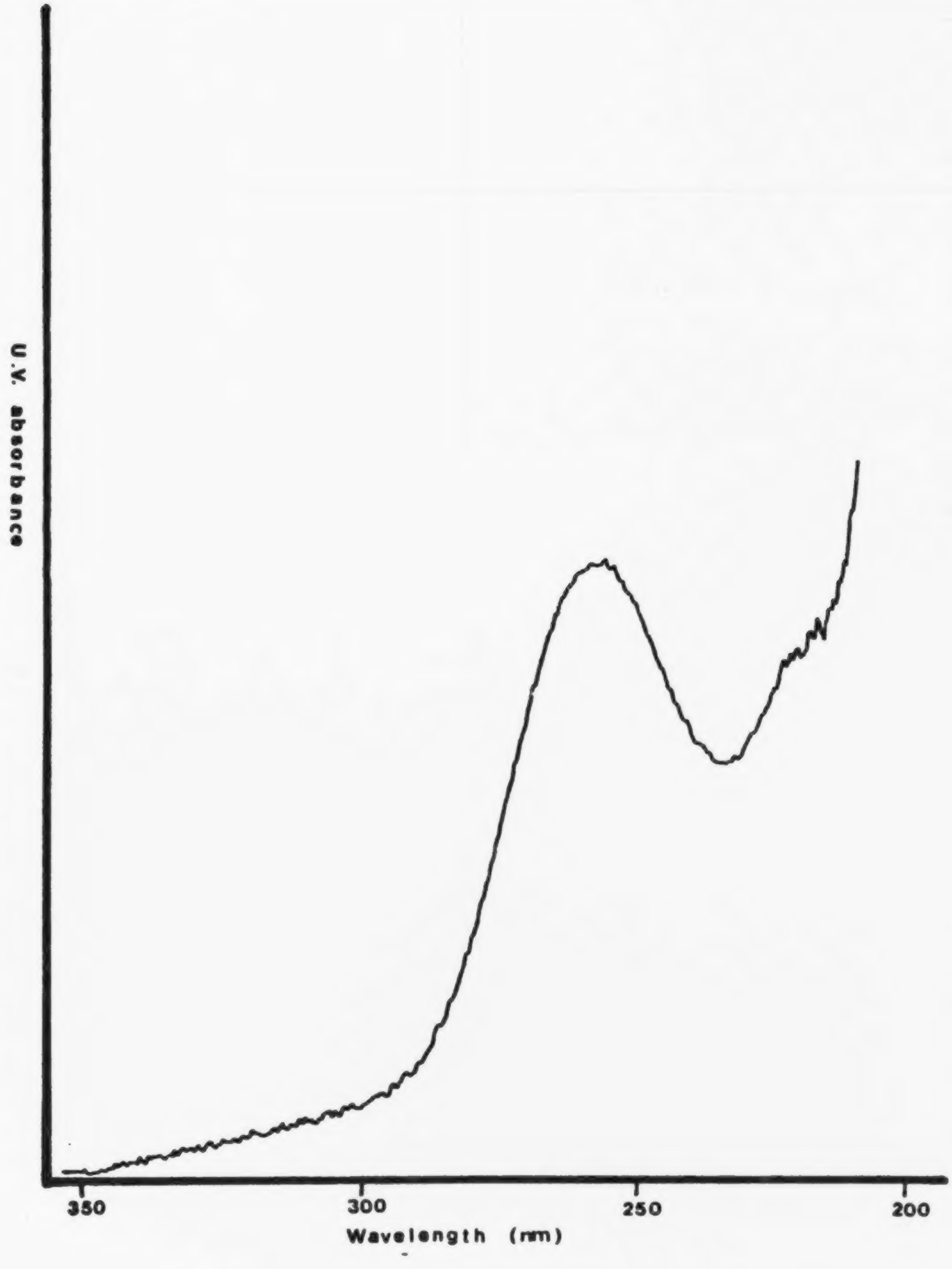


Figure 88. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: uracil.

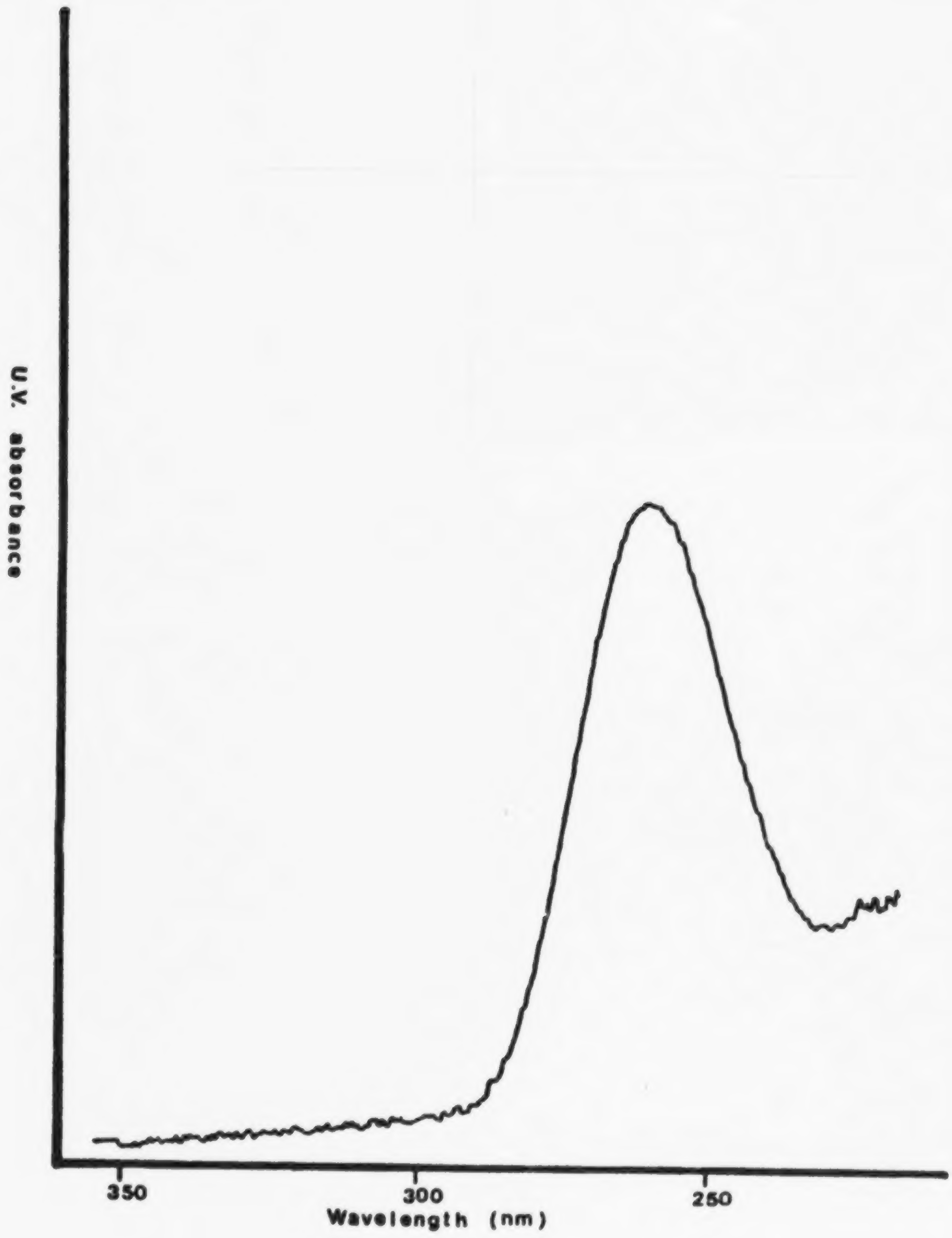


Figure 89. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: thymine.

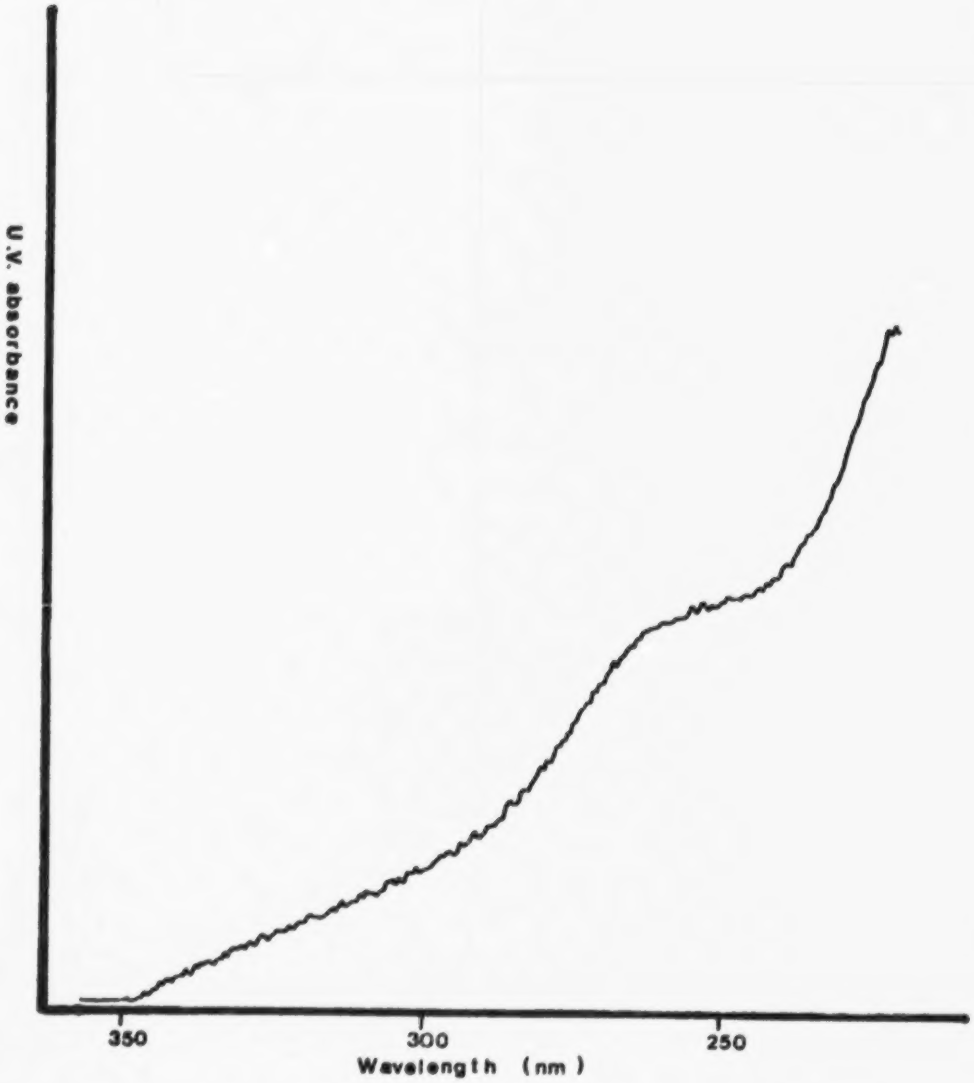


Figure 90. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: adenine.

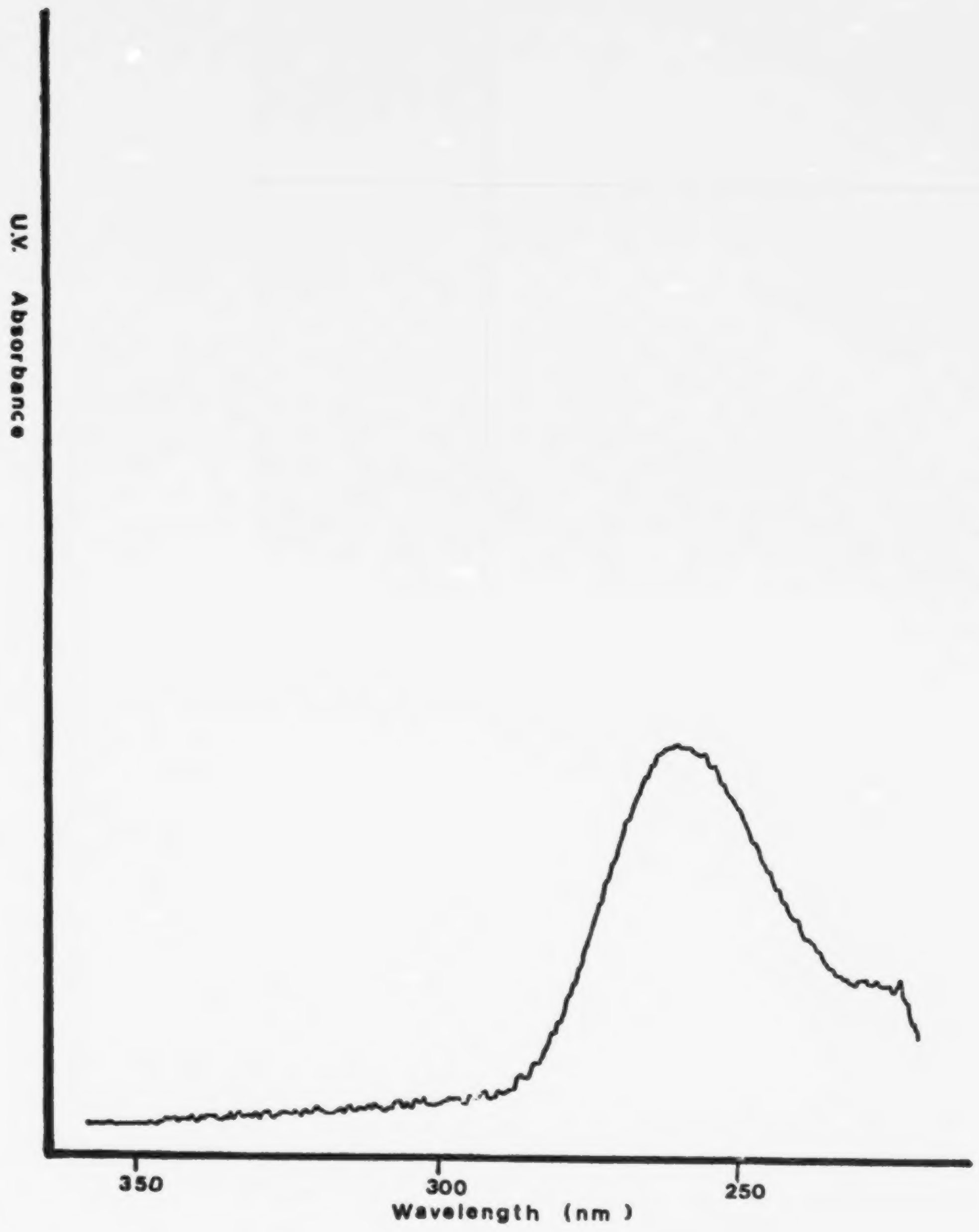


Figure 91. Middleton Pond, Danvers, MA (raw; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: purine.

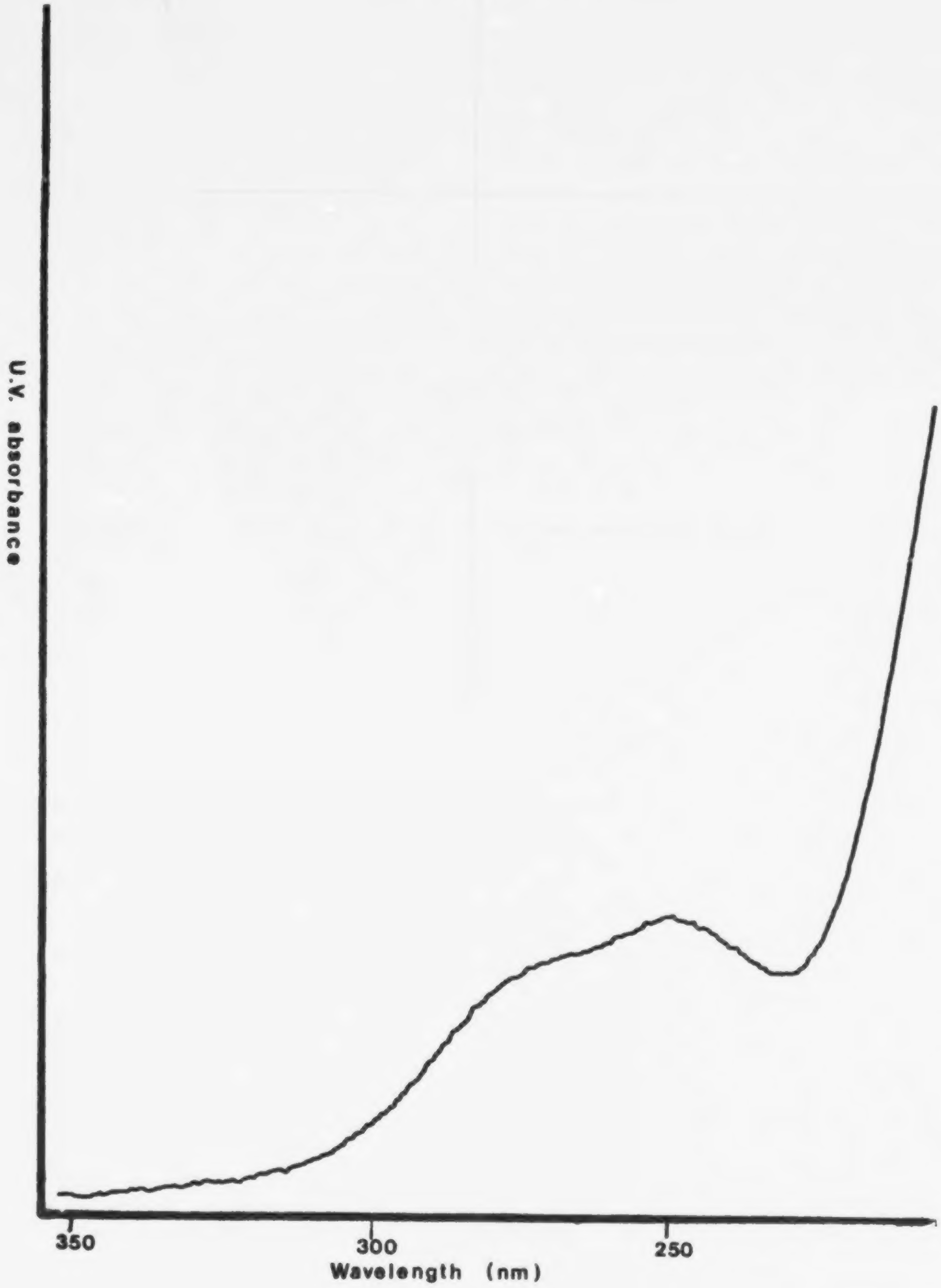


Figure 92. Middleton Pond, Danvers, MA (resin filtered; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: guanine.

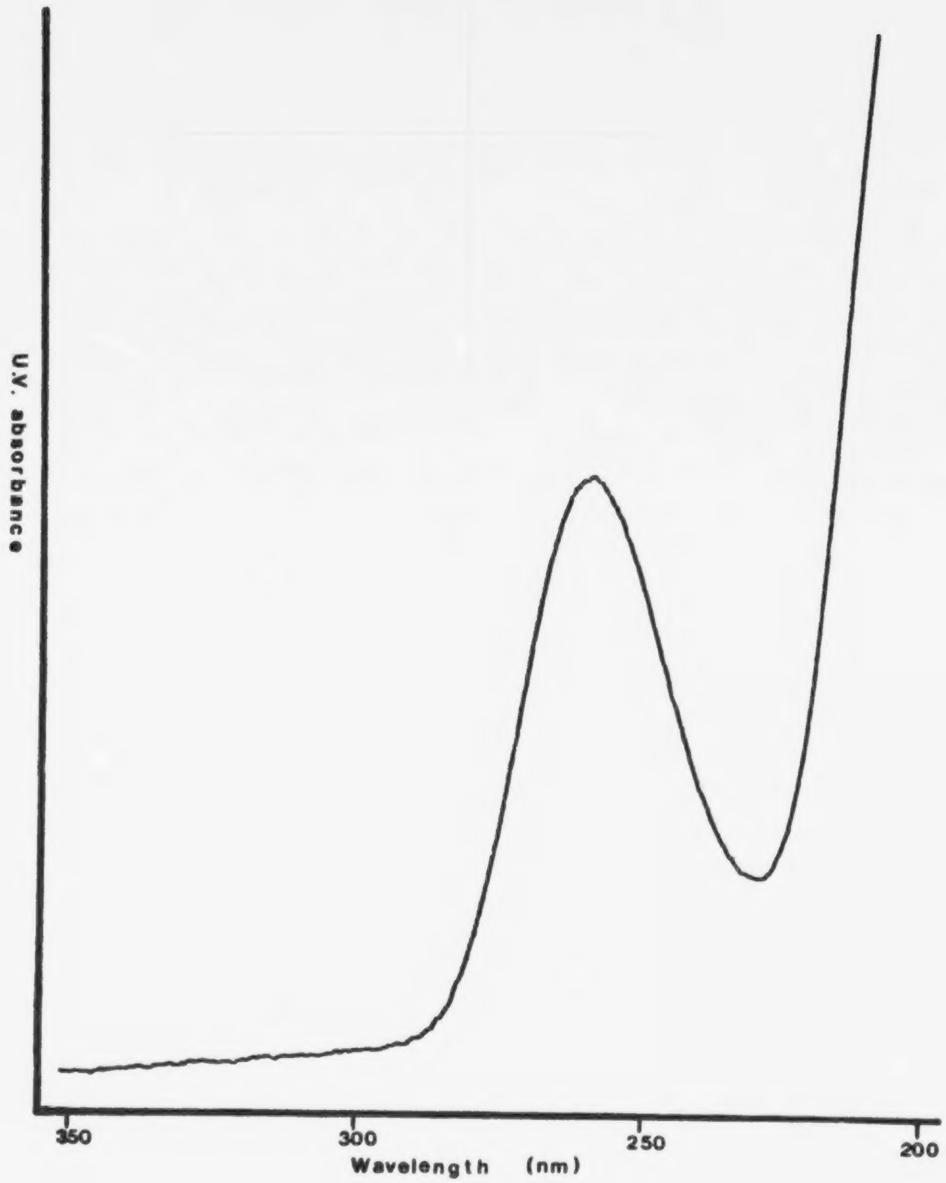


Figure 93. Middleton Pond, Danvers, MA (resin filtered; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: thymine.

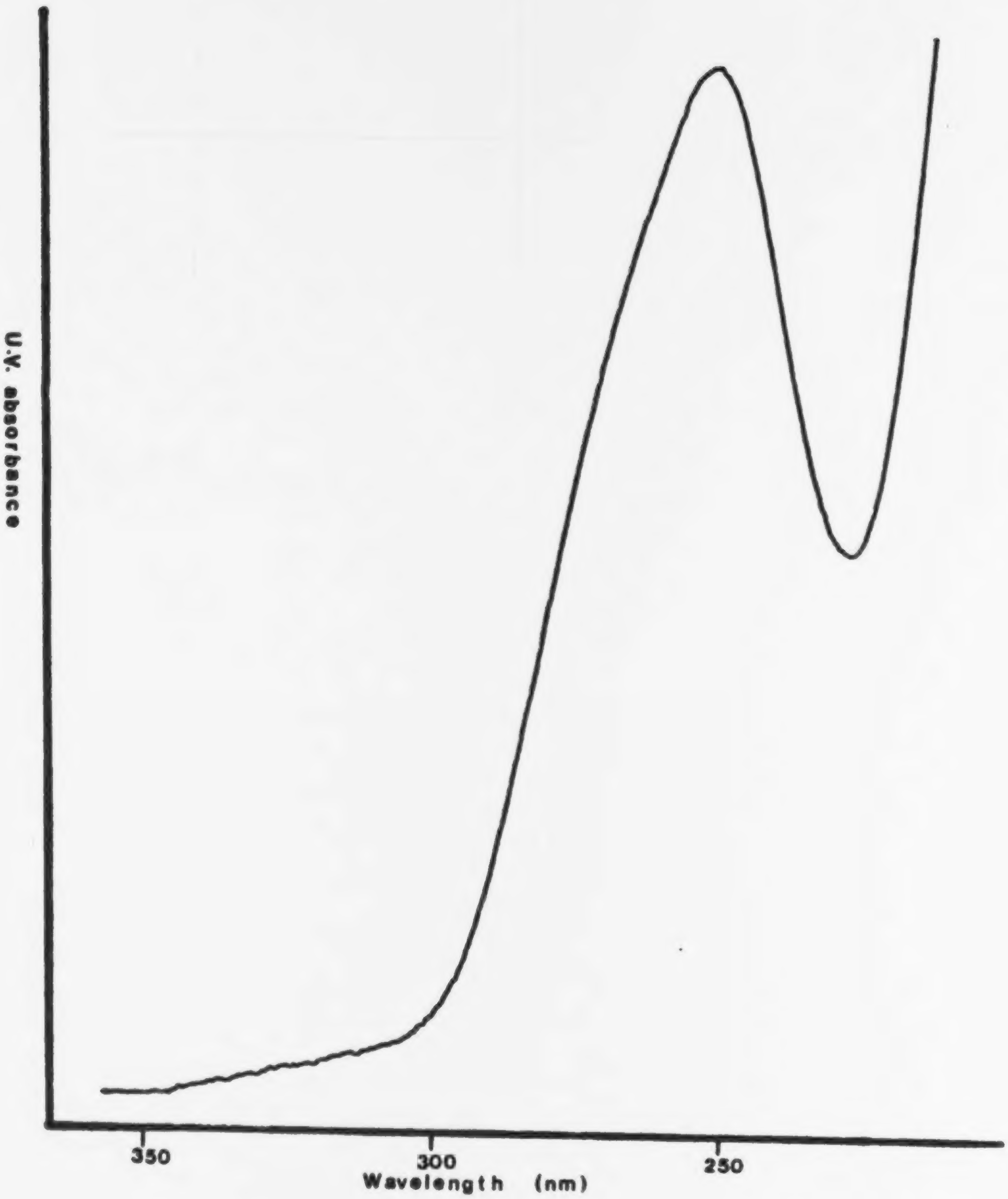


Figure 94. Middleton Pond, Danvers, MA (resin filtered; x 1,000; 7-11-78). Borate buffered eluant, suspected compound: uracil.

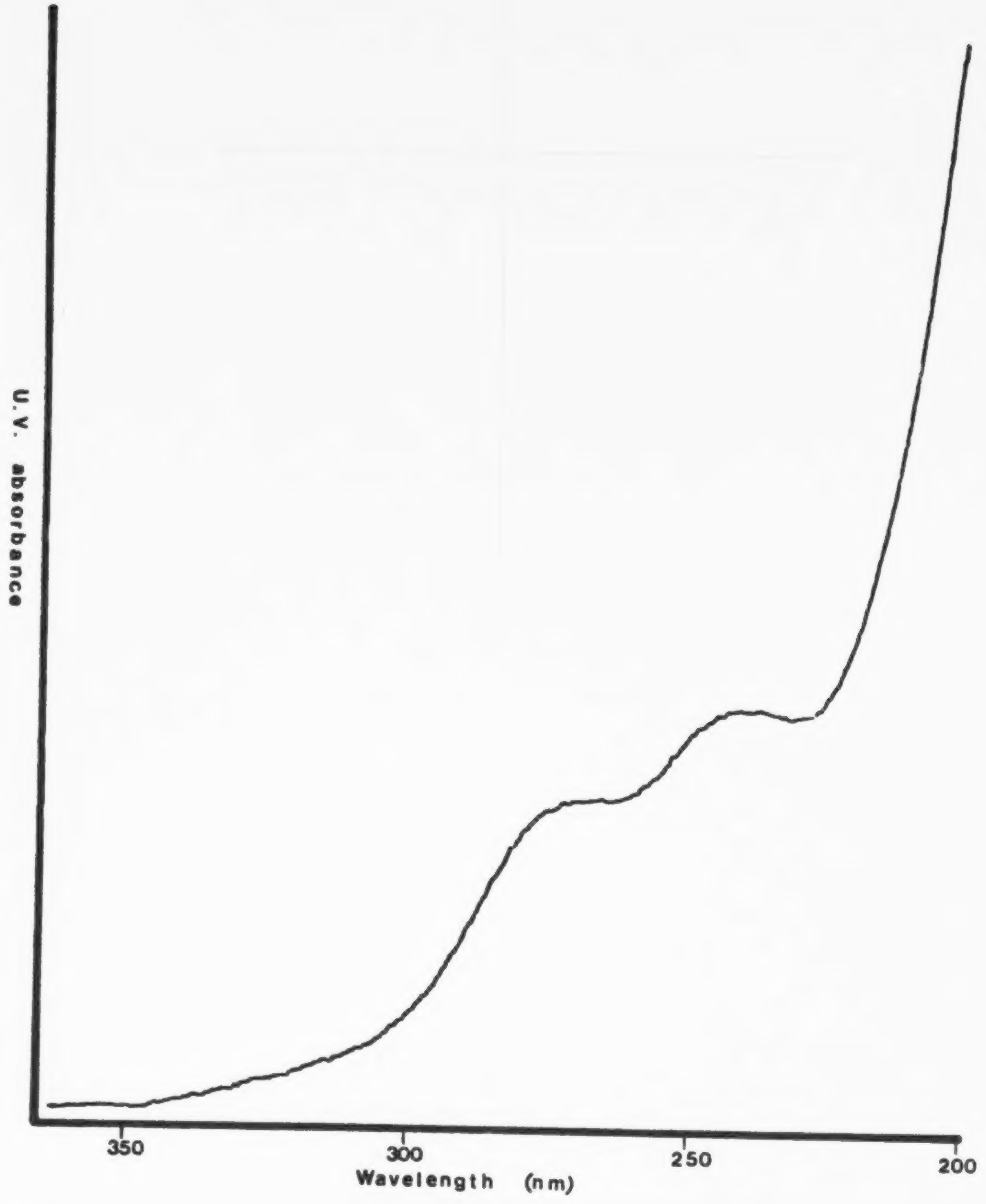


Figure 95. Concord River, Billerica, MA (resin filtered, x 2,000, 6-9-78). Borate buffered eluant, suspected compound: guanine.

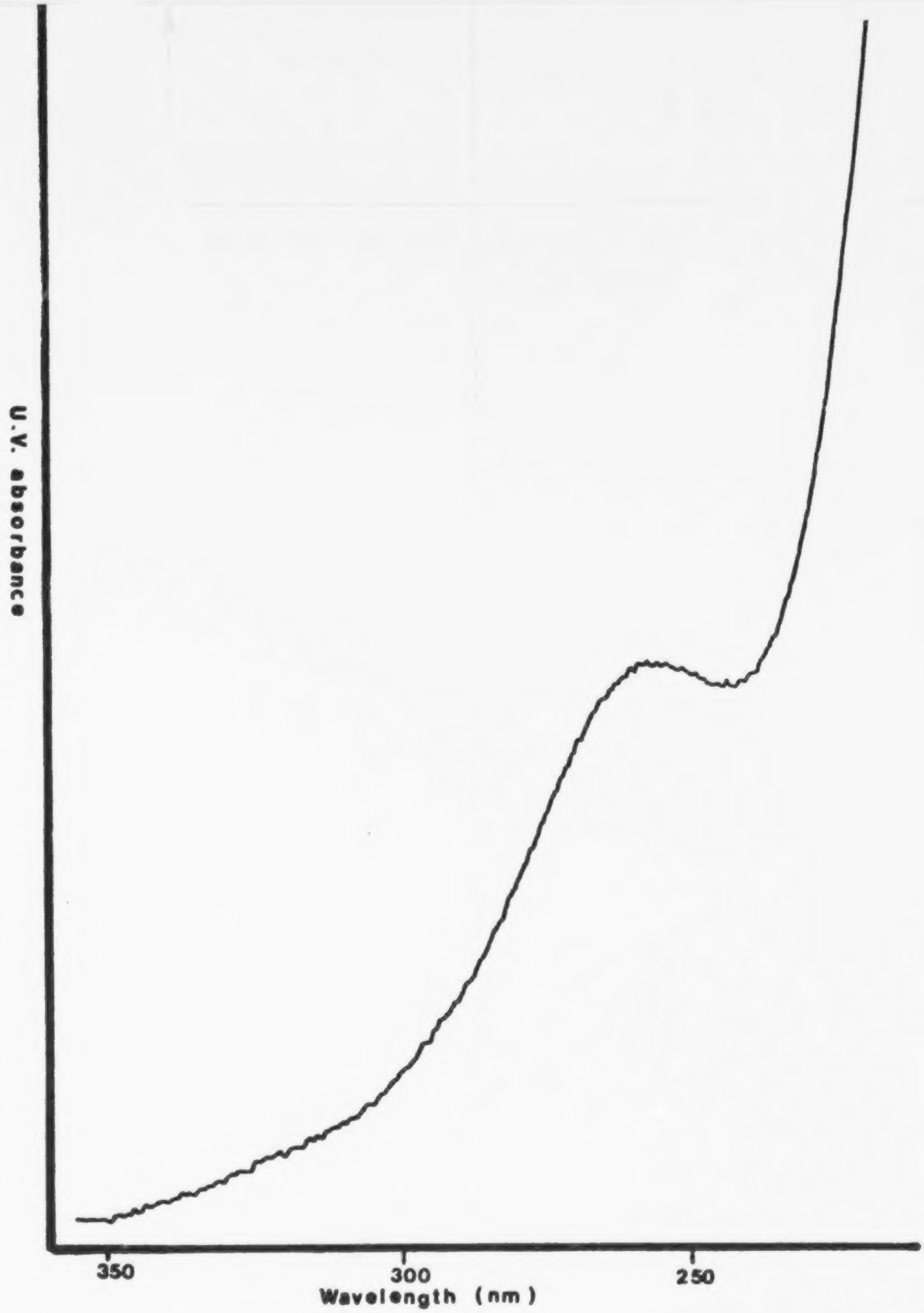


Figure 96. Concord River, Billerica, MA (resin filtered; x 1,000; 7-4-78). Borate buffered eluant, suspected compound: uracil.

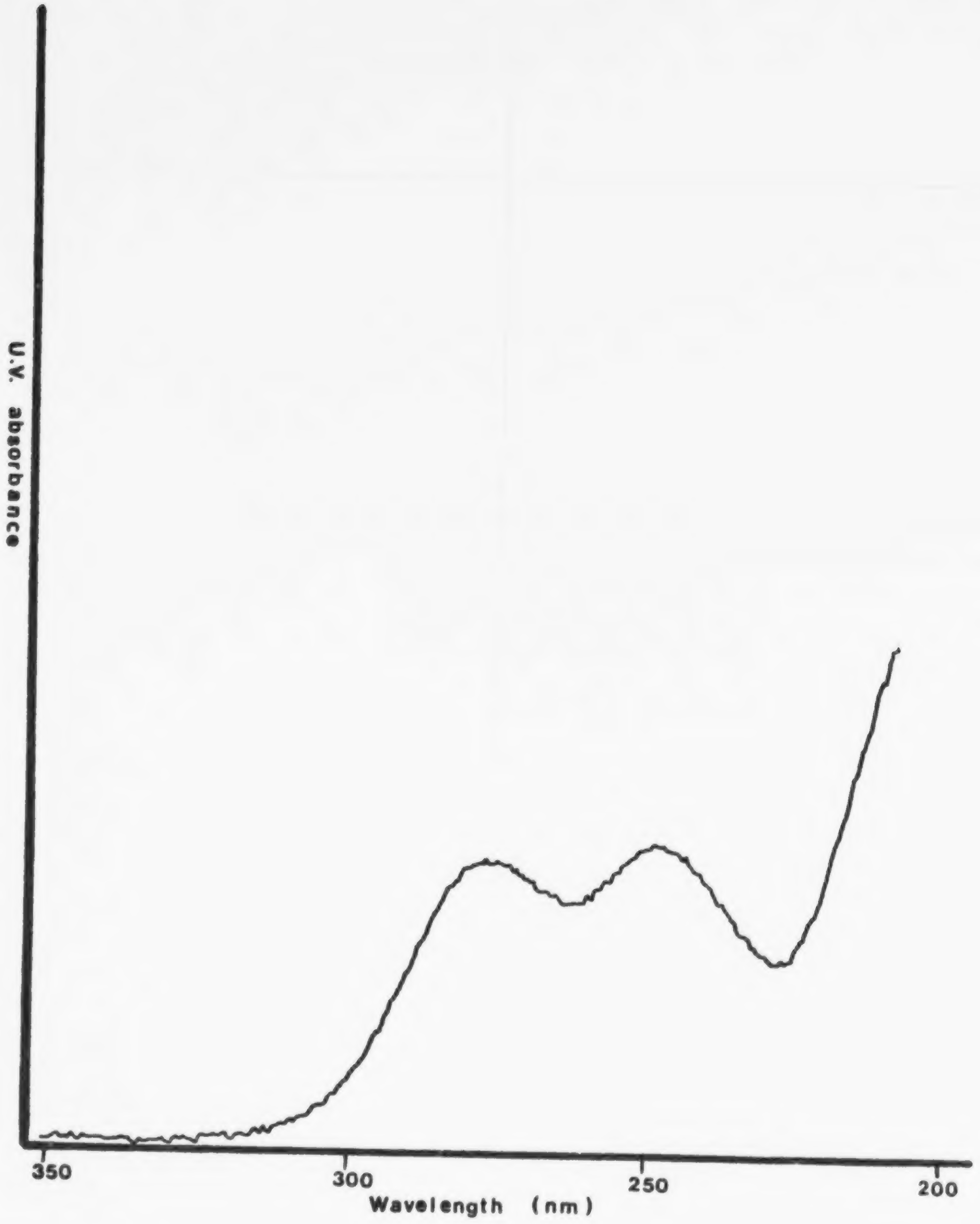


Figure 97. Concord River, Billerica, MA (resin filtered; x 1,000; 7-4-78). Borate buffered eluant, suspected compound: guanine.

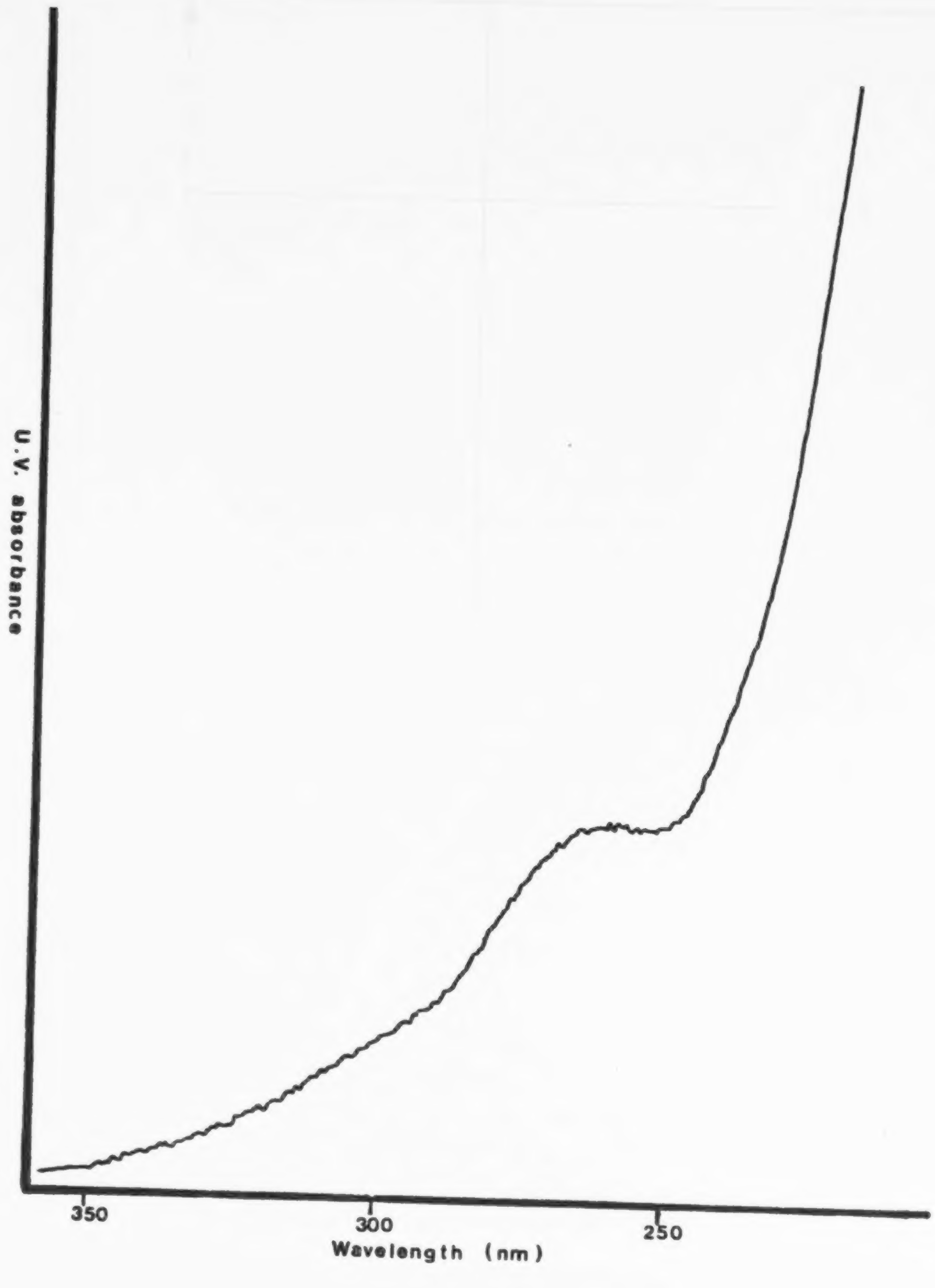


Figure 98. Concord River, Billerica, MA (resin filtered; x 1,000; 7-4-78). Borate buffered eluant, suspected compound: adenine.

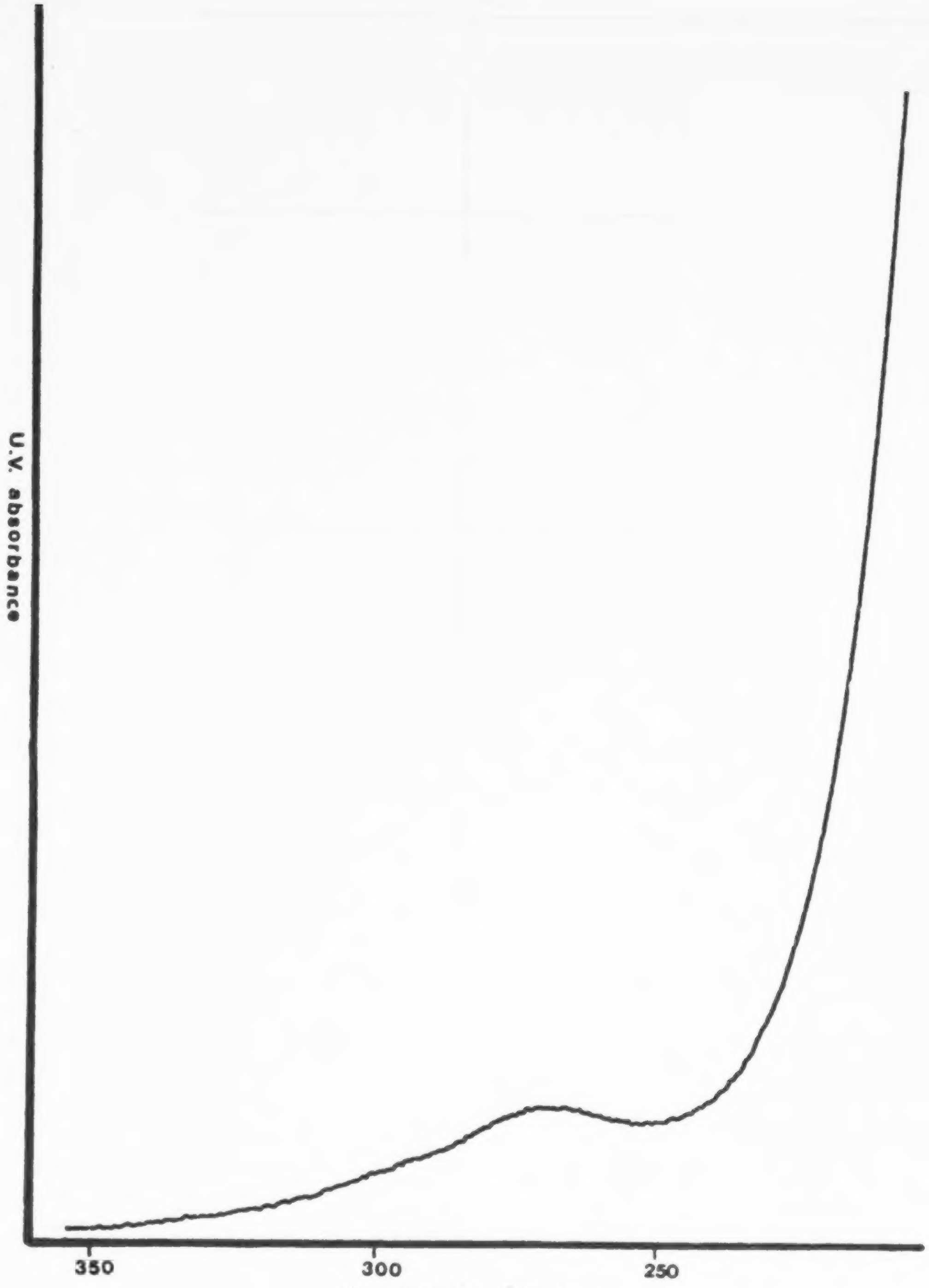


Figure 99. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: cytosine.

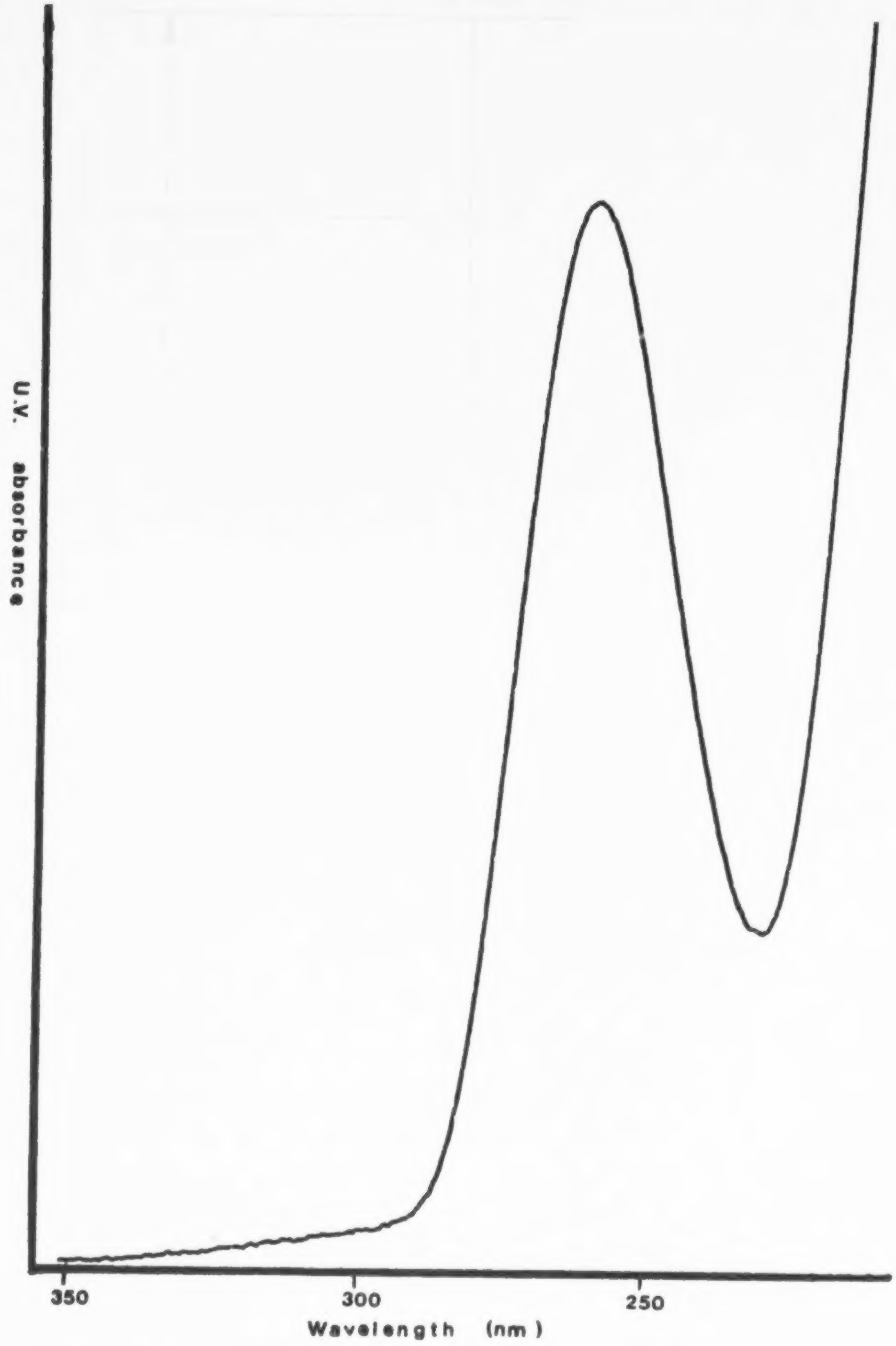


Figure 100. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: uracil.

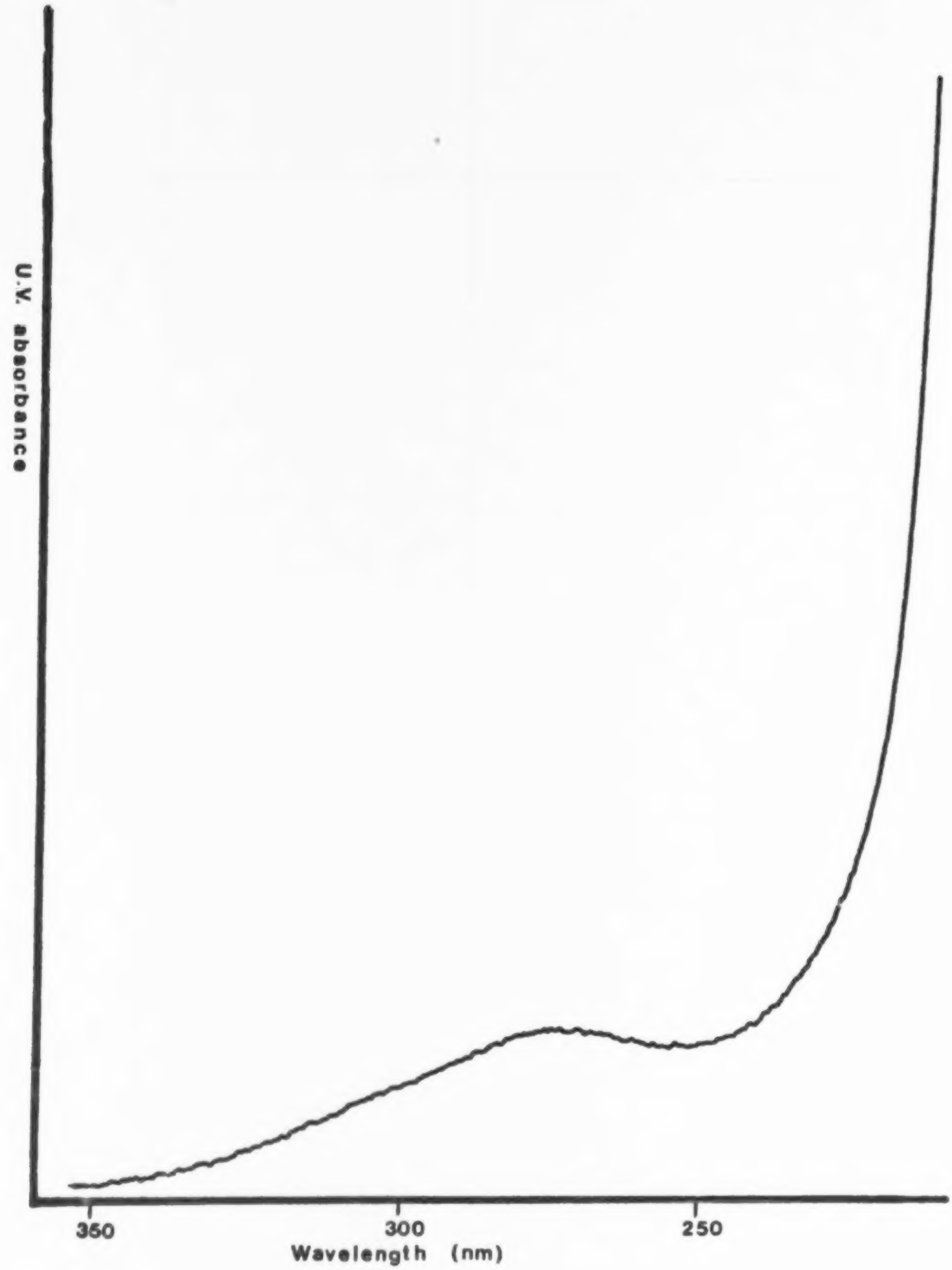


Figure 101. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: 5-chlorouracil.

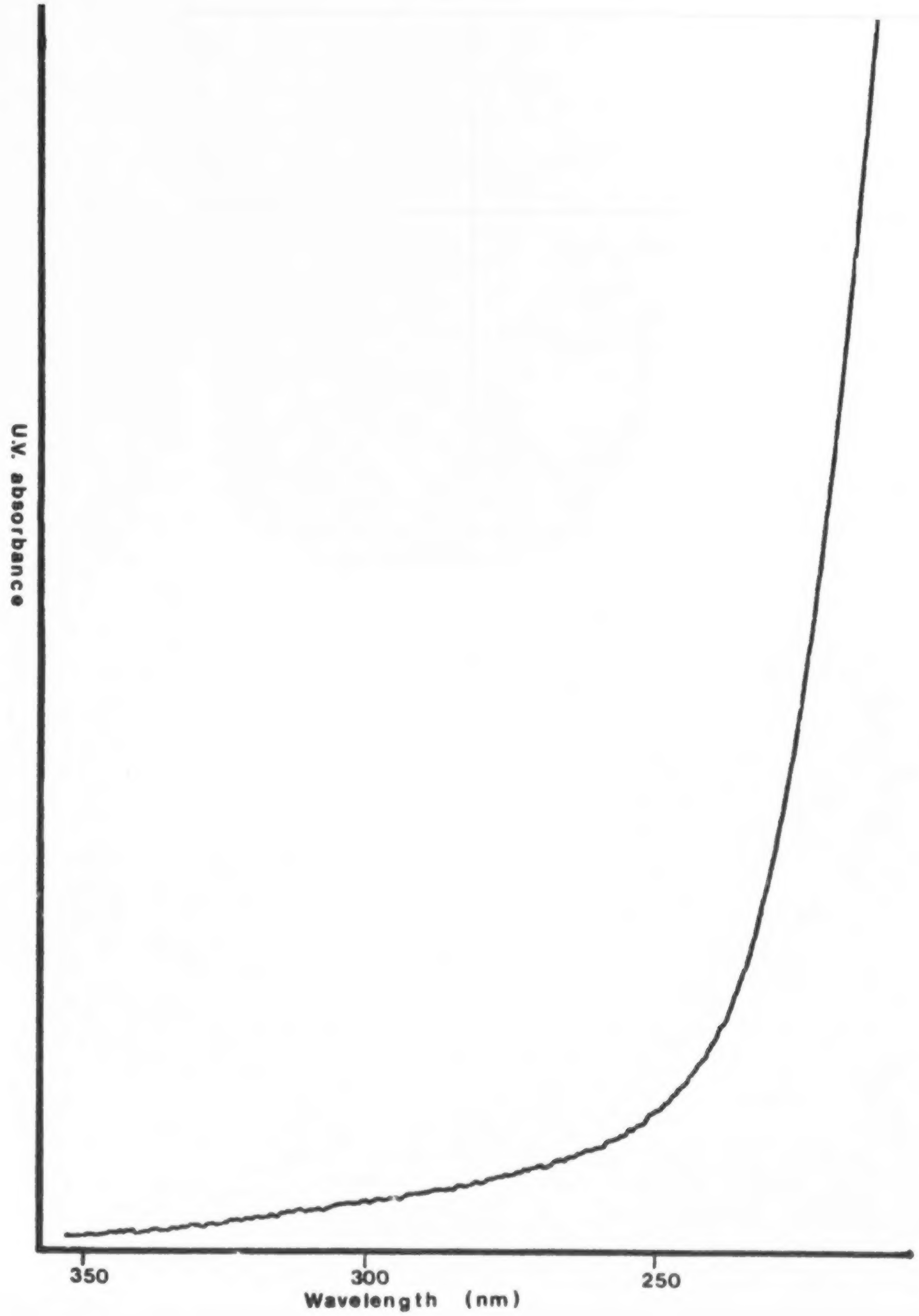


Figure 102. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: pyrrole.

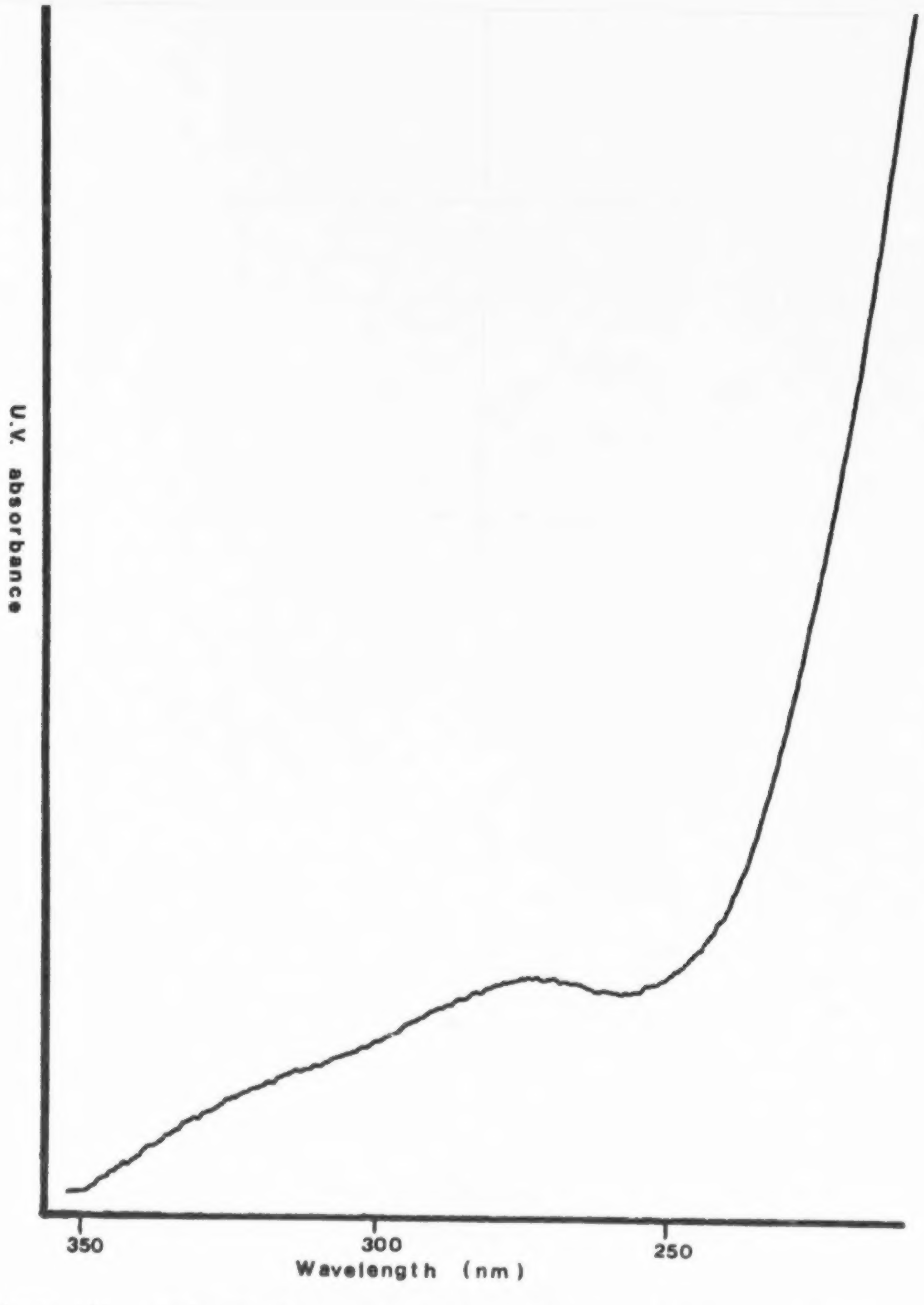


Figure 103. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: tryptophan.

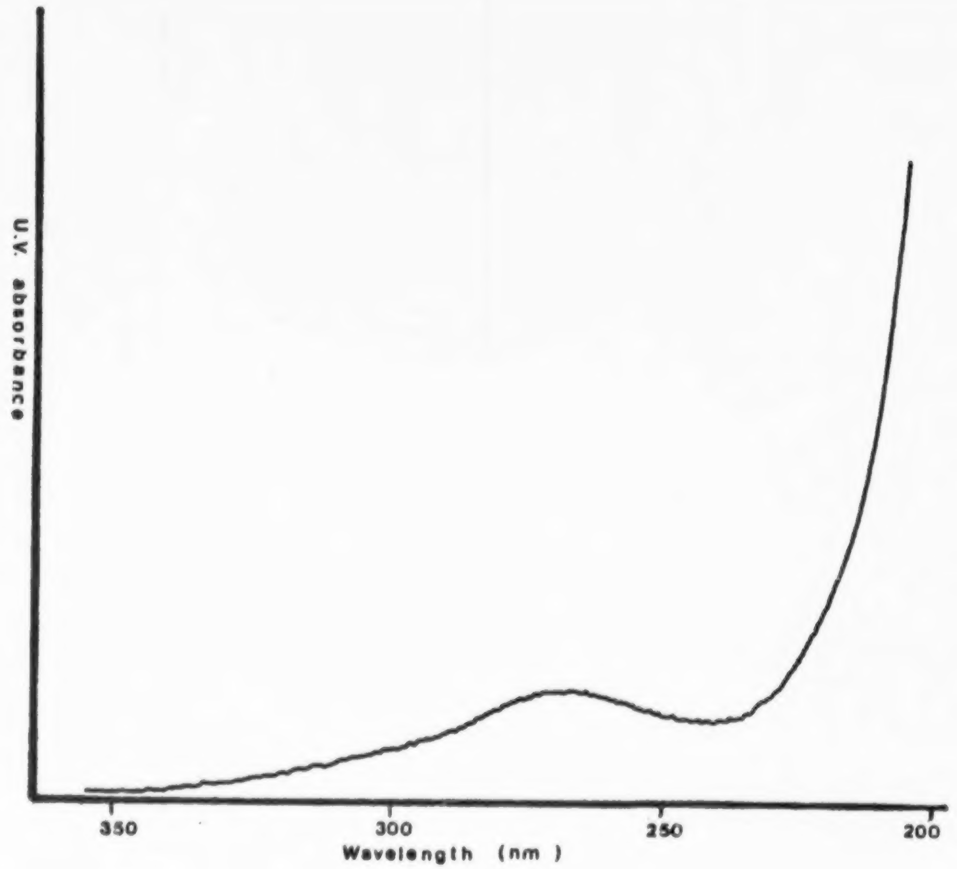


Figure 104. *Anabaena flos aquae* (resin filtered; x 1,615). Phosphate buffered eluant, suspected compound: thymine.

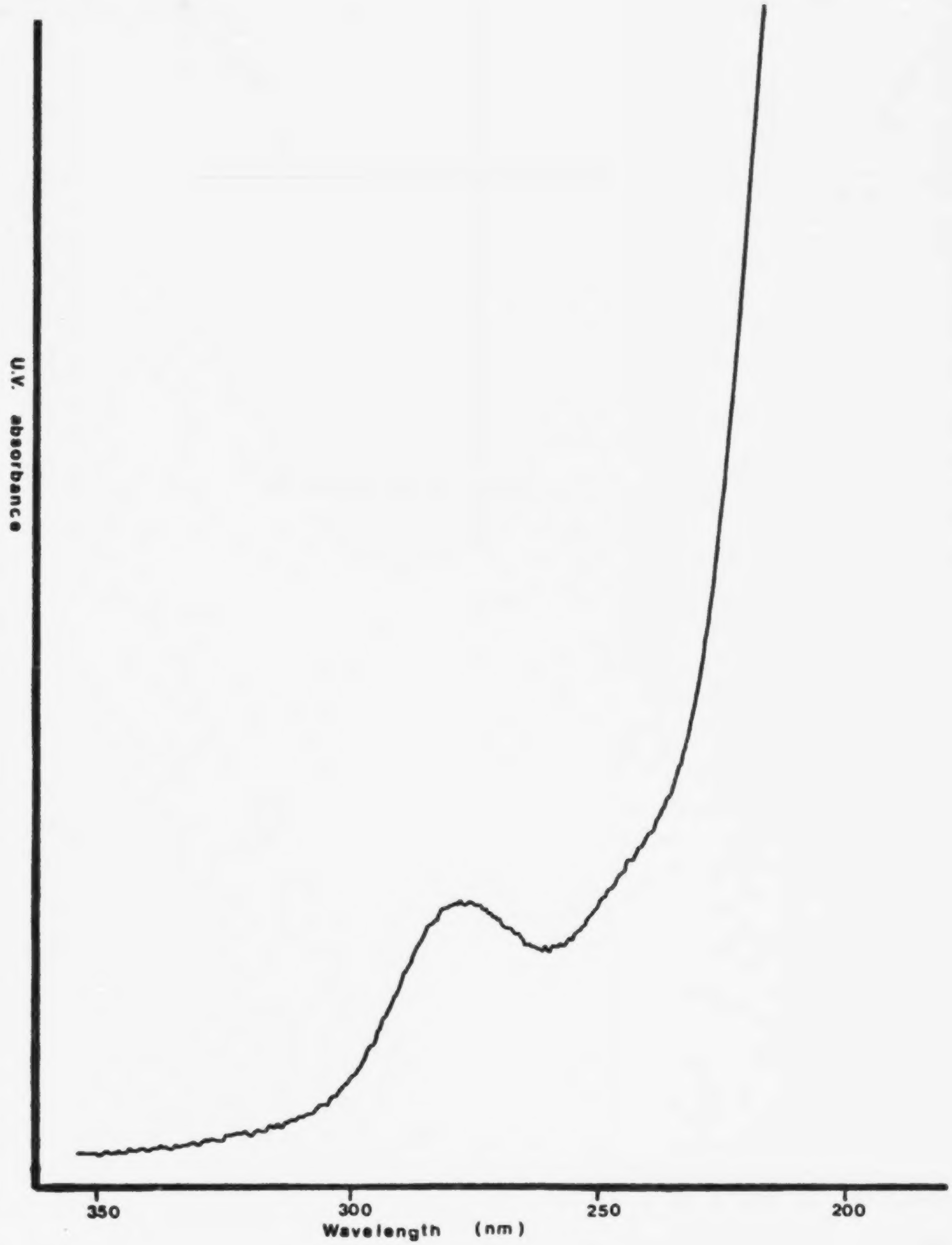


Figure 105. *Anabaena flos aquae* (raw; x 1,750). Borate buffered eluant, suspected compound: tyrosine.

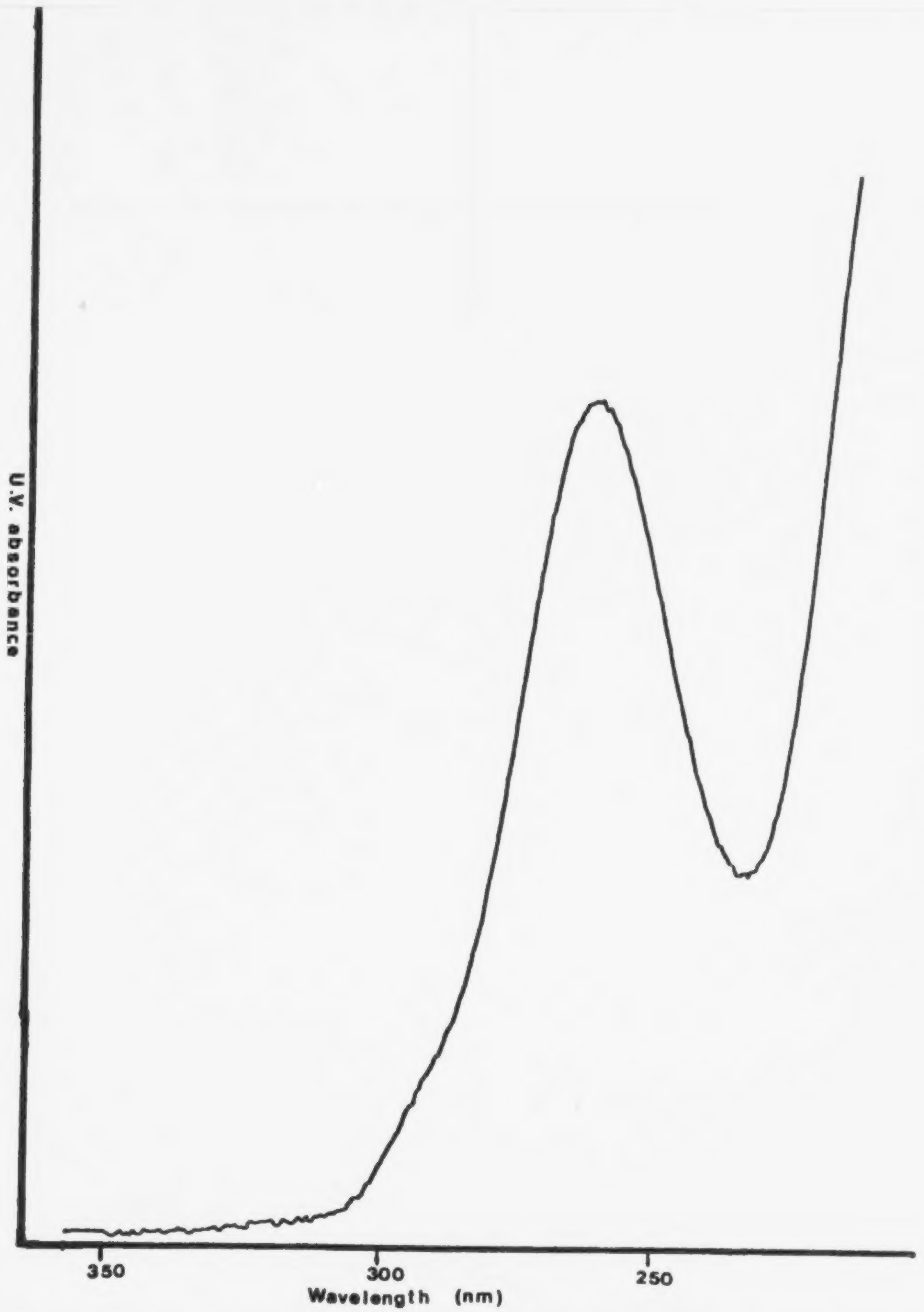


Figure 106. *Anabaena flos aquae* (raw; x 1,750). Borate buffered eluant, suspected compound: uracil.

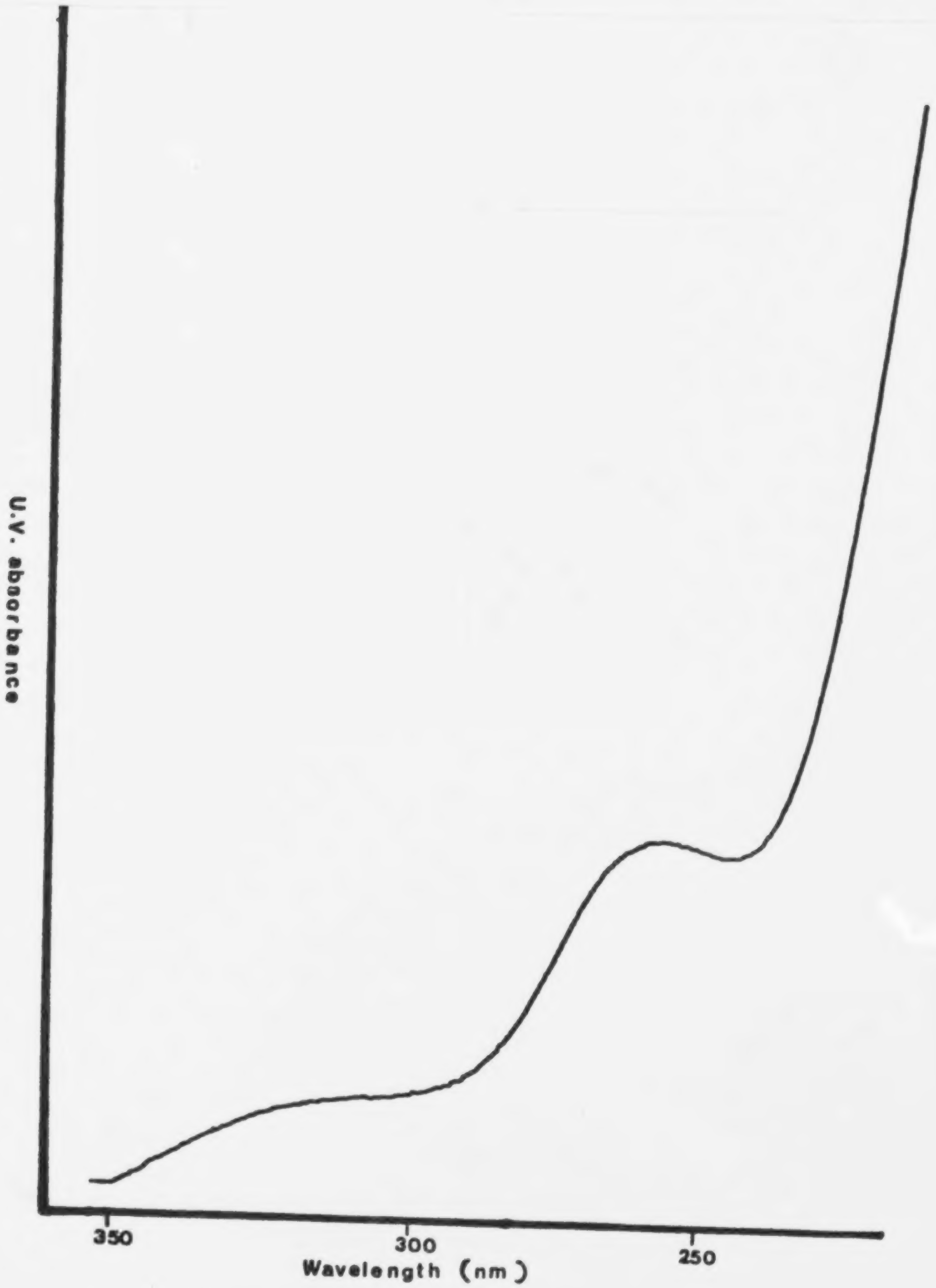


Figure 107. *Oscillatoria tenuis* (resin filtered; x 1,630). Borate buffered eluant, suspected compound: adenine.

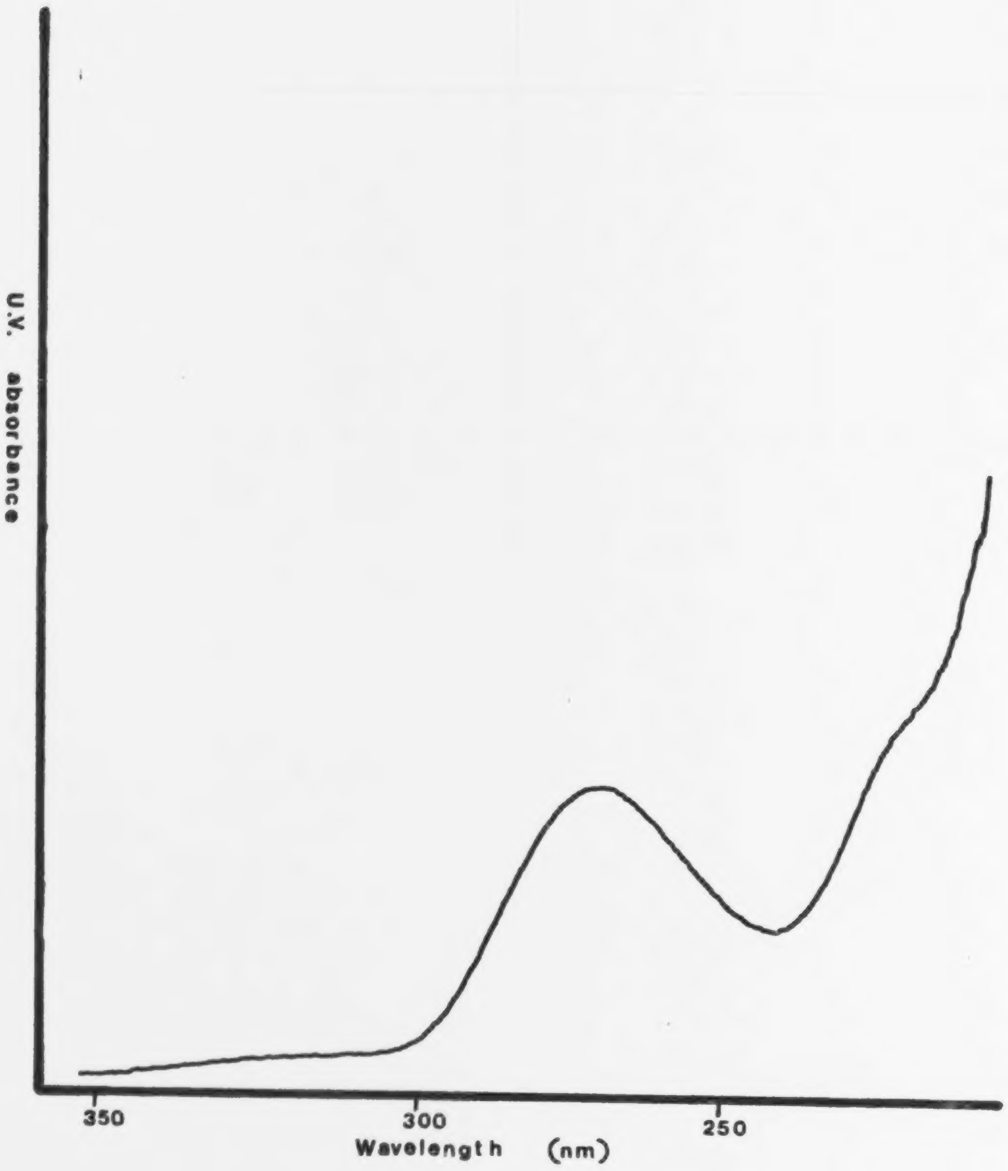


Figure 108. *Oscillatoria tenuis* (resin filtered; x 1,630). Borate buffered eluant, suspected compound: purine.

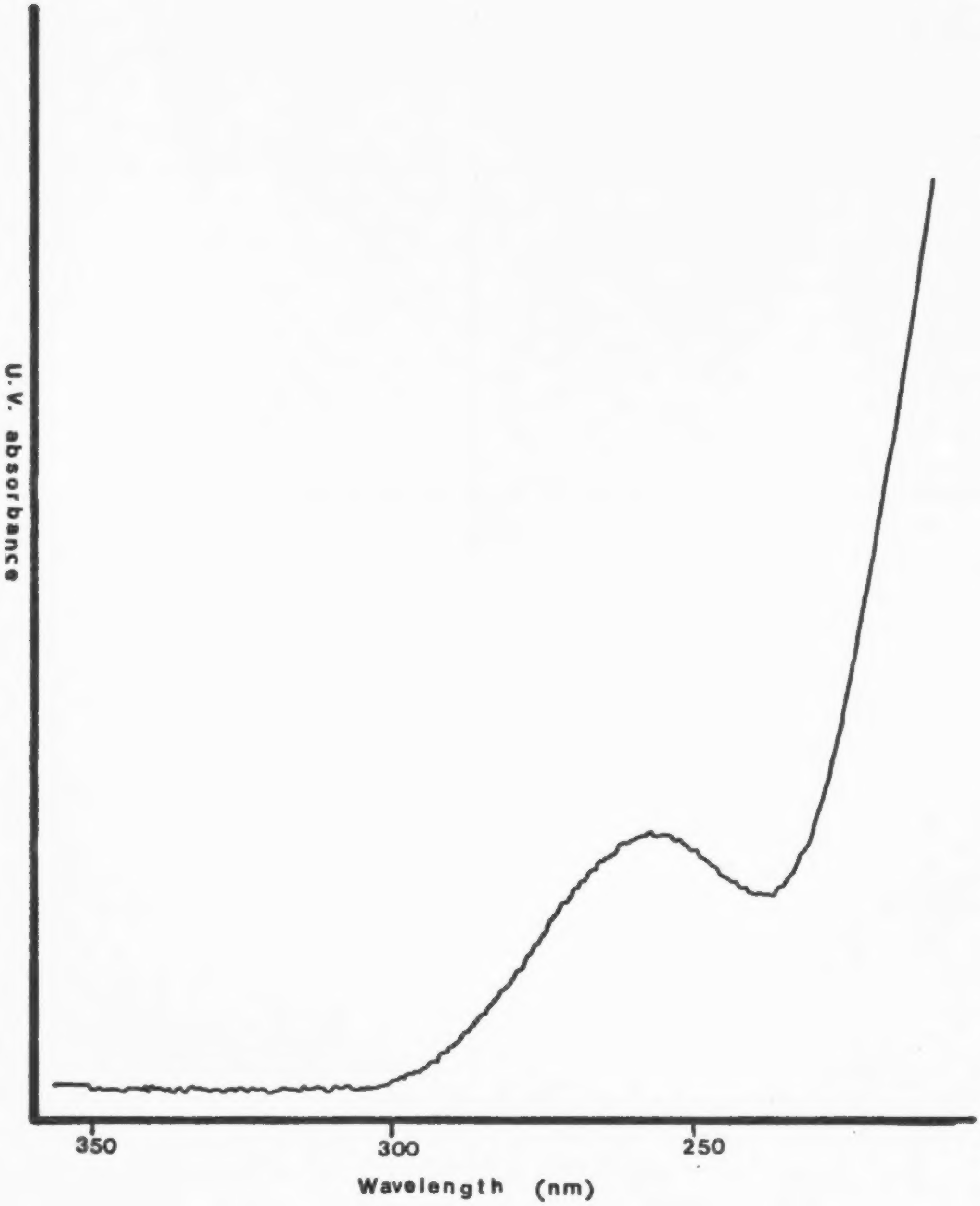


Figure 109. *Oscillatoria tenuis* (raw; x 1,860). Borate buffered eluant, suspected compound: uracil.

TABLE 29. U.V. ADSORBANCE DATA FOR COMPOUNDS RESOLVED ON ZORBAX C8 USING 0.05 M PHOSPHATE OR .05 M BORATE BUFFERED ELUANT TO MeOH GRADIENTS

suspected compound	λ_{peak}	similarity to reference compound[a]	λ_{trough}	similarity to reference compound	$\frac{A_{peak}}{A_{trough}}$ [c]	$\frac{\left(\frac{A_{peak}}{A_{trough}}\right)_{sample}}{\left(\frac{A_{peak}}{A_{trough}}\right)_{reference}}$ [c]	$\frac{A_{220}}{A_{233}}$ [c]	$\frac{\left(\frac{A_{220}}{A_{233}}\right)_{sample}}{\left(\frac{A_{220}}{A_{233}}\right)_{reference}}$ [c]
I. Middleton Pond, Danvers, MA, raw, 7/11/78 (phosphate buffered eluant)								
uracil	260	+++[b]	231	+++	1.30	0.33	0.87	0.67
5-chlorouracil	273	+++	250	+	1.57	0.48	1.48	0.52
thymine	261	+++	230	+++	-	-	1.55	0.70
adenine	262	+++	232	+++	2.11	0.43	1.86	1.29
adenine	256	+++	232	+++	-	-	1.61	1.12
purine	267	+++	245	0	1.58	0.32	-	-
Ia. Middleton Pond, Danvers, MA, raw, 7/11/78 (borate buffered eluant)								
uracil	256	+++	234	+++	1.60	0.55	1.24	0.95
thymine	261	+++	231	+++	3.0	1.03	1.14	0.50
adenine	260	+++	247	0	-	-	-	-
purine	261	++	230	+++	2.36	0.56	1.0	0.42
II. Middleton Pond, Danvers, MA, resin filtered, 7/11/78 (borate buffered eluant)								
guanine	276 & 250	+++	263 & 230	+++	-	-	0.92	0.66
thymine	260	+++	230	+++	2.87	0.99	1.67	0.73
uracil	253	++	227	+++	1.7	0.59	1.3	0.97
III. Middleton Pond, Danvers, MA, entrainment precipitation method, 7/11/78 (phosphate buffered eluant)								
cytosine	263	+++	247	+++	1.07	0.77	1.83	1.36
adenine	260	+++	230	+++	1.88	0.58	1.52	1.05

(continued)

TABLE 29 (continued)

suspected compound	λ_{peak}	similarity to reference compound[a]	λ_{trough}	similarity to reference compound	$\frac{A_{peak}}{A_{trough}}$ [c]	$\frac{\left(\frac{A_{peak}}{A_{trough}}\right)_{sample}}{\left(\frac{A_{peak}}{A_{trough}}\right)_{reference}}$ [c]	$\frac{A_{220}}{A_{233}}$ [c]	$\frac{\left(\frac{A_{220}}{A_{233}}\right)_{sample}}{\left(\frac{A_{220}}{A_{233}}\right)_{reference}}$ [c]
IV. Concord River, Billerica, MA, resin filtered, 6/9/78 (borate buffered eluant)								
guanine	272 & 241	+++[b]	264 & 231	+++	-	-	1.15	0.82
V. Concord River, Billerica, MA, resin filtered, 7/4/78 (borate buffered eluant)								
uracil	257	+++	243	+++	1.15	0.36	1.70	1.31
guanine	279 & 249	+++	262 & 227	+++	-	-	1.92	1.36
adenine	262	+++	252	0	-	-	2.02	1.56
VI. Anabaena flos aquae, resin filtered (phosphate buffered eluant)								
cytosine	268	+++	250	+++	1.13	0.81	2.33	1.73
uracil	257	+++	229	+++	3.21	0.79	1.52	1.19
5-chlorouracil	272	+++	243	+++	1.10	0.33	1.85	0.64
pyrrole	flat	-	-	-	-	-	-	-
tryptophan	273	++	256	0	1.07	0.40	2.04	0.33
thymine	266	+++	239	++	1.36	0.41	2.12	0.97
VII. Anabaena flos aquae, raw (borate buffered eluant)								
tyrosine	272	+++	260	0	-	-	2.27	0.99
uracil	260	+++	233	+++	2.33	0.80	2.04	1.57
VIII. Oscillatoria tenuis, resin filtered, x 1630 (borate buffered eluant)								
adenine	259	+++	245	0	-	-	2.03	1.56
purine	271	+++	242	+	1.82	0.46	1.79	0.74
IX. Oscillatoria tenuis, raw, x 1860 (borate buffered eluant)								
uracil	256	+++	237	+	1.29	0.44	2.33	1.79

[a] see Table 27

[b] +++ = ± 5 nm

++ = ± 7 nm

+ = ± 10 nm

0 = >|10|nm

[c] A = absorbance value at wavelength shown

TABLE 30. SAMPLES RESULTING IN EITHER POORLY RESOLVED OR UNIDENTIFIABLE CHROMATOGRAPHIC PEAKS

I. Samples concentrated by low temperature distillation followed by lyophilization:

1. Surface impoundment, Bethesda, Ohio (resin-filtered)
2. Middleton Pond, Danvers, MA, 6/21/78 (resin-filtered at both pH 2.0 and pH 7.0)
3. Merrimack River, Lawrence, MA, 6/14/78 (resin-filtered)

All of the samples exhibited a large unresolved group of compounds rapidly eluting from the column. A corresponding fluorescamine-fluorescence peak was frequently observed. Underivatized fluorescence for this rapidly eluting group of compounds was substantially less than the corresponding derivatized fluorescence indicating the presence of primary amine compounds in the concentrated samples. The total concentration of this group of compounds was later calculated to be greater than about an equivalent of 40 mg/L of glycine. Retention data of reference amino acid compounds (Table 25) additionally suggested that this rapidly eluting group of compounds may have consisted of primary amine materials.

The chromatograms display both a U.V. and fluorescence trace for most samples. The U.V. ordinate is in absorbance units. A full scale reading is equal to the sensitivity value reported in each chromatogram. The fluorescence ordinate is calibrated by both a sensitivity and range setting. Fluorescence settings equal to a sensitivity of 40.0% and 45.0% and a range of 0.2 for each, resulted in a full scale reading for 36 mg/L and 12 mg/L fluorescamine derivatized glycine, respectively. Every 10% increase in the sensitivity setting approximately doubled the fluorometric sensitivity.

The recording pens for the U.V. and fluorescence traces are displaced from each other by 1.3 cm as indicated on the abscissa of the chromatograms. Corresponding U.V. and fluorescence peaks are separated by only 1.2 cm because of the lag between the U.V. and fluorescence detectors.

Chromatograms of raw and resin-filtered samples are included for most sites. The same compounds were identified at some sites in both the raw and resin-filtered samples, while other compounds were observed in only one of the differently treated samples. No significant improvement in the chromatographic baseline was observed for any of the resin-filtered samples.

Table 31 shows the concentrations of organic nitrogen compounds identified in the concentrated field and laboratory samples. Concentrations were calculated from peak heights of known concentrations of reference compounds according to the equation:

TABLE 31. CONCENTRATIONS OF ORGANIC NITROGEN COMPOUNDS IDENTIFIED IN CONCENTRATED FIELD AND LABORATORY SAMPLES[a]

Compound	Middleton Pond[b], Danvers, MA (raw) 7/11/78	Middleton Pond[b], Danvers, MA (resin filtered) 7/11/78	Middleton Pond[b], Danvers, MA (entrainment precipitation method 7/11/78	Concord River Billerica, MA (resin filtered) 6/9/78	Concord River, Billerica MA (raw) 7/4/78	*Anabaena flos aquae* (raw)	*Anabaena flos aquae* (resin filtered)	*Oscillatoria tenuis* (raw)	*Oscillatoria tenuis* (resin filtered)
adenine	860 (445)		90 (46)		150 (77)				40 (21)
5-chlorouracil	60 (11)						10 (2)		
cytosine			20 (8)				40 (16)		
guanine		190 (88)		140 (64)	170(79)				
purine	200 (93)								40 (19)
pyrrole							160 (33)		
thymine	240 (53)	90 (20)					40 (9)		
tryptophan							30 (4)		
tyrosine						340 (26)			
uracil	250 (62)	170 (42)			60 (15)	100 (25)	80 (20)	20 (5)	
organic nitrogen (μg N/L)	21.7×10^3	21.3×10^3	-	0.34×10^3	1.84×10^3	2.24×10^3	1.60×10^3	1.78×10^3	1.31×10^3
% of organic nitrogen identified in sample	3.1	0.7	-	18.8	9.3	2.3	6.2	0.2	3.1

Values out of parentheses indicate compound concentrations as μg/L

[a] Values in parentheses are calculated organic nitrogen concentrations (as μg N/L)

[b] Sampled during the occurrence of blue-green algal bloom

$$\begin{array}{c}\text{concentration}\\ \text{of suspected}\\ \text{compound}\end{array} = \left[\begin{array}{l}\text{peak height of}\\ \text{suspected com-}\\ \text{pound at 220 nm}\\ \text{(in chart units)}\end{array} \times \frac{\text{absorbance units}}{\text{chart unit}} \right] \div \begin{array}{l}\text{absorbance units}\\ \text{at 220 nm per}\\ \text{mg/L reference}\\ \text{compound}\end{array} \tag{10}$$

Absorbance values (220 nm) per mg/L of reference compound for materials separated using either the phosphate or borate buffered eluant are shown in Tables 32 (see page 180) and 33, respectively.

TABLE 33. VALUES FOR CONVERTING PEAK HEIGHTS OF NITROGENOUS COMPOUNDS RESOLVED ON ZORBAX C8 TO CONCENTRATION IN mg/L (BORATE BUFFERED ELUANT)

Compound	concentration (mg/L)	absorbance at 220 nm (absorbance units)	absorbance at 220 nm per mg/L of compound (absorbance units)[a]
barbituric acid	20	0.150	.0075
ℓ-histidine	610	0.230	.0004
cytosine	15	0.100	.0067
5-chlorouracil	60	0.700	.0117
uracil	10	0.060	.0060
creatinine	10	0.140	.0140
guanine	50	0.086	.0017
thymine	75	0.280	.0037
pyrrole	100	0.090	.0009
purine	75	0.230	.0031
adeine	100	0.170	.0017
tryptophan	50	0.220	.0044
pyrimidine	40	0.250	.0063
tyrosine	20	0.056	.0028

[a] Absorbance at 220 nm per mg/L of compound = [absorbance at 220 nm/concentration of compound (mg/L)]

Middleton Pond, sampled during the occurrence of a blue-green algal bloom (Figures 58 to 63) and the laboratory grown culture of *Anabaena flos aquae* (Figures 72 to 74) exhibited the greatest number of identifiable nitrogenous compounds. 5-chlorouracil, thymine, and uracil were identified in both of these samples. Adenine, guanine, and purine were identified in Middleton Pond and not in the *Anabaena* sample, while the reverse was found for cytosine, pyrrole, and tryptophan.

Comparison of raw and resin-filtered samples at the same collection sites, showed that both different and some of the same compounds were identified in the dissimilarly treated samples. One treatment did not appear to favor the identification of a larger number of compounds. The total number

TABLE 32. VALUES FOR CONVERTING PEAK HEIGHTS OF NITROGENOUS COMPOUNDS RESOLVED ON ZORBAX C8 TO CONCENTRATION IN mg/L (PHOSPHATE BUFFERED ELUANT)

compound	concentration (mg/L)	Absorbance at 233 nm (absorbance units)	$\frac{\text{absorbance at 220 nm}}{\text{absorbance at 233 nm}}$[a]	absorbance at 220 nm per mg/L of compound (absorbance unit)[b]
barbituric acid	20	0.066	1.22	.0040
ℓ-histidine	600	0.074	1.72	.0002
cytosine	10	0.072	1.35	.0097
creatinine	10	0.066	0.66	.0044
guanine	50	0.068	1.28	.0006
5-chlorouracil	50	0.058	2.87	.0033
thymine	75	0.084	2.23	.0025
pyrrole	100	0.062	5.13	.0032
purine	75	0.108	0.82	.0012
tryptophan	50	0.144	6.09	.0175
pyrimidine	40	0.102	0.36	.0009
indole	90	0.086	8.40	.0080

[a] Absorbance ratios were determined from U.V. spectral scans of reference compounds. (Figures 17 to 30)

[b] Absorbance at 220 nm per mg/L of compound = [(absorbance at 233 nm) × (absorbance at 220 nm/absorbance at 233 nm)]/(concentration of compound in mg/L)

of compounds identified at a given collection site was maximized by analyzing both raw and resin-filtered samples.

Concentrations of organic nitrogen compounds ranged from 860 μg/L of adenine in Middleton Pond (7/11/78) to 10 μg/L of 5-chlorouracil in the raw laboratory grown culture of *Anabaena flos aquae*. The average values for the compounds identified in the water supply samples were 367, 60, 20, 167, 200, 110, and 160 μg/L of compounds, for adenine, 5-chlorouracil, cytosine, guanine, purine, thymine and uracil, respectively. Uracil, adenine and guanine were found most frequently in the water supply samples while 5-chlorouracil, cytosine and purine were only encountered once in these sources. A total of 7 N-organic compounds were identified in the water supplies while 9 compounds were found in the laboratory grown blue-green algal cultures. Pyrrole, tryptophan and tyrosine were found in the algal cultures and not in the water supplies. Guanine was found in the water supplies but not in the algal cultures.

The percent of organic nitrogen identified in each sample was calculated according to the equation:

$$\begin{array}{c}\text{\% of organic nitrogen}\\ \text{identified in sample}\end{array} = \frac{\begin{array}{c}\text{sum of organic nitrogen concentrations}\\ \text{of individual compounds identified in}\\ \text{sample } (\mu\text{g N/L})\end{array}}{\begin{array}{c}\text{total organic nitrogen } (\mu\text{g/L})\\ \text{determined in sample}\end{array}} \,. \qquad (11)$$

The total organic nitrogen was determined by Kjeldahl-N analysis. Organic nitrogen concentrations of individual compounds identified in the samples were calculated according to the equation:

$$\begin{array}{c}\text{organic nitrogen}\\ \text{concentration of}\\ \text{individual compound}\\ \text{identified in}\\ \text{sample } (\mu\text{g N/L})\end{array} = \mu\text{g compound/L} \times \frac{\mu\text{M compound}}{\mu\text{g compound}} \times \frac{\mu\text{M nitrogen}}{\mu\text{M compound}} \times \frac{14\ \mu\text{g nitrogen}}{\mu\text{M nitrogen}} \,. \qquad (12)$$

The percent of organic nitrogen identified in the water supplies and blue-green algal cultures ranged from 0.7% to 18.8% (mean = 8%) and 0.2% to 6.3% (mean = 3%), respectively. The majority of organic nitrogen in these samples, therefore, was not characterized.

The types of compounds identified included: amino acids (tyrosine and tryptophan), nucleic acid bases (adenine, cytosine, guanine, and uracil), purines (adenine, guanine, purine), and pyrimidines (cytosine, thymine, and uracil). Three of these substances, uracil, pyrrole, and tryptophan, were previously shown to be precursors for the formation of trihalomethanes (89, 38). Purines and pyrimidines were shown to be intermediates for compounds causing mutagenic activity in finished waters upon chlorination (90-96, 64). 5-chlorouracil was shown to be mutagenic (30,97).

Significance of Findings

The types and concentrations of compounds identified in municipal water supplies are environmentally significant because of their ability to yield interference or false positive tests in determining free chlorine, and to be precursors of trihalomethanes during chlorination. Several trihalomethane precursors were found in the municipal water supply samples and in laboratory-grown blue-green algal cultures: pyrrole, thymine, tryptophan, and uracil. The molar yield of chloroform of these materials was previously determined in laboratory studies at pH 7 by Baum (10) and are shown in Table 34. Molar yield is defined as: (moles of chloroform formed)/(moles of compound used).

TABLE 34. HIGHEST CONCENTRATION OF CHLOROFORM PRODUCED AT pH 7[a]

compound	time (hours)	molar yield of $CHCl_3$(%)[b]
pyrrole	0.3	0.7
thymine	3.4	0.5
tryptophan[c]	24.0	7.8
tryptophan (pH = 7.5)[d]	7.0	17.9
uracil	0.7	0.6

[a] Initial chlorine concentration = 9.0×10^{-5} M; HOCl/compound ratio = 9:1 except where noted; after Baum (10).

[b] $\%\text{ molar yield of } CHCl_3 = \frac{\text{(moles of chloroform formed)}}{\text{(moles of compound used)}} \times 100$

[c] Initial chlorine concentration = 10×10^{-5} M; HOCl/compound ratio = 10:1

[d] Initial chlorine concentration = 10×10^{-5} M; HOCl/compound ratio = 20:1

Table 35 shows the calculated levels of chloroform produced by chlorination of these compounds in samples studied. It was assumed that the N-organic compounds yielded chloroform according to the values shown in Table 34, and that they would not be removed prior to chlorination of the water supplies. The calculated total production of chloroform for all the samples was well below the proposed maximum contaminant level of 0.1 mg/L (100 ppb) for total trihalogenated methanes (73).

The molar yield of chloroform, however, is highly pH dependent. The molar yield of tryptophan, for example, increases from 7.8% to pH 7, to 17.9% at pH 7.5, 24% at pH 8, and about 31% at pH 9. The molar yield of uracil

TABLE 35. CALCULATED LEVELS OF $CHCl_3$ PRODUCED IN WATER SAMPLES CONTAINING N-ORGANIC SUBSTANCES UPON CHLORINATION (AT pH 7)[a]

site	compound	concentration (μM/L)	chloroform produced[b] (μM/L)	chloroform produced (μg/L)
Middleton Pond Danvers, MA 7/11/78	thymine uracil	1.90 2.23	0.010 0.013	1.26 1.46
Concord River Billerica, MA 7/4/78	uracil	0.54	0.003	0.36
Anabaena flos aquae culture	uracil tryptophan	0.89 0.15	0.005 0.011	0.60 2.34
Oscillatoria tenuis culture	uracil	0.18	0.001	0.12

[a] Proposed maximum contaminant level = 100 μg/L chloroform.

[b] Chloroform produced (μM/L) = concentration (μM/L) × molar yield of $CHCl_3$ (%)

increases from 0.6% at pH 7 to about 8% at pH 8 and about 40% at pH 9. One hundred percent molar yield of chloroform is obtained for uracil above about pH 10. The molar yield for pyrrole increases (after about 24 hours contact time) from 0.5% at pH 7.5 to 7.6% at pH 9.1. Some of these values were obtained by allowing mixtures to react at neutral or slightly acidic pH values for several hours with subsequent increase of pH by addition of NaOH. The reader is referred to Baum's thesis (10) for a full discussion of these reactions. The chloroform which could be produced by the N-organic compounds present in the water supplies and laboratory blue-green algal cultures under more alkaline conditions are shown in Table 36. The calculated levels of chloroform formed under the more alkaline conditions in the Middleton Pond and Concord River samples are more than 10% of the proposed maximum contaminant level for total trihalomethanes and are therefore significant. Additional N-organic compounds not identified in the dissolved N-organic fraction of these samples might additionally contribute to the level of $CHCl_3$ production. The increased molar yield of $CHCl_3$ in alkaline waters stresses the need to carefully monitor the pH of water supplies in treatment facilities and to provide increased organic contaminant removal capabilities in alkaline waters.

The significance of the levels of N-organic materials identified in the water supplies can also be evaluated by calculating the amount of combined chlorine which may be formed during chlorination. As discussed previously,

TABLE 36. CALCULATED LEVELS OF $CHCl_3$ PRODUCED IN WATER SUPPLIES CONTAINING N-ORGANIC SUBSTANCES UPON CHLORINATION (AT HIGHER pH VALUES[a]

site	compound	chloroform produced[b] (μg/L)
Middleton Pond Danvers, MA 7/11/78	thymine	NC[c]
	uracil	20.0 (pH 8) 99.9 (pH 9)
Concord River Billerica, MA 7/4/78	uracil	4.8 (pH 8) 24.2 (pH 9)
Anabaena flos aquae culture	uracil	8.0 (pH 8) 39.9 (pH 9)
	tryptophan	5.5 (pH 7.5) 7.4 (pH 8) 9.5 (pH 9)
Oscillatoria tenuis culture	uracil	1.6 (pH 8) 8.1 (pH 9)

[a] Proposed maximum contaminant level = 100 μg/L chloroform

[b] Chloroform produced (μg/L) =

$$\left|\begin{array}{c}\text{concentration of compound}\\ (\mu M/L)\end{array}\right| \times \left|\begin{array}{l}\text{molar yield of } CHCl_3 \text{ at}\\ \text{particular pH value (\%)}\end{array}\right| \times \frac{\mu g \text{ compound}}{\mu M \text{ compound}}$$

[c] NC: not calculated because data on % chloroform yield at higher pH values was unavailable.

combined chlorine is a much less active disinfectant than free chlorine. The combined forms, however, tend to react similarly with many analytical reagents for active chlorine. When these N-chloro compounds are formed, tests for free chlorine may indicate a non-existent bactericidal or virucidal behavior. Table 37 shows the calculated values of combined chlorine that could be formed during chlorination. The total conversion of N-organic constituents to combined forms and a 1:1 molar reaction are assumed.

Chlorine residuals of 0.2 to 1 mg/L after 15 to 30 minues of contact time generally result in 99.9% destruction of *Escherichia coli* (1) (an indicator species for pathogenic bacteria) in drinking water. A 15 minute free chlorine residual of 0.5 mg/L is generally taken as an acceptable level of disinfection (1). If this residual is comprised of the less germicidal combined forms, however, the water supply may not be hygienically safe. The calculated levels of combined forms of chlorine yielding falsely positive

TABLE 37. CALCULATED VALUES OF COMBINED CHLORINE FORMED UPON CHLORINATION

sample	compound	molar concentration (μM/L)	active chlorine concentration[a] (mg/L)
Middleton Pond Danvers, MA 7/11/78)	adenine	6.36	0.452
	5-chlorouracil	0.41	0.029
	purine	1.67	0.119
	thymine	1.90	0.135
	uracil	2.23	0.158
	total......................................		0.982
Concord River Billerica, MA 6/9/78	guanine	0.93	0.066
Concord River Billerica, MA 7/4/78	adenine	1.11	0.79
	guanine	1.12	0.80
	uracil	0.54	0.38
	total		0.197

[a] active chlorine concentration =

$$[\text{concentration of compound } (\mu M/L)] \times \frac{\text{71 } \mu g \text{ chlorine determined } \underline{\text{as free aqueous chlorine}}}{\mu M \text{ combined chlorine}} \times 10^{-3} \text{ mg}/\mu g$$

tests for free aqueous chlorine in the Middleton Pond sample and in one sample from the Concord River were calculated to be 0.982 and 0.197 mg/L free aqueous chlorine, respectively. Assumed free chlorine residuals of 0.5 mg/L in the finished drinking water from these sources, then, might not provide adequate disinfection, since a significant portion of this value may be comprised of the less germicidal combined forms.

The finding of trihalomethane (THM) precursors at levels which could produce significant amounts of chloroform upon chlorination under alkaline pH conditions, and the demonstration that the N-organic constituents identified could lead to false positive tests for free chlorine stresses the environmental significance of the findings of this study. A subcommittee of the National Research Council recently reported (98) that it was virtually impossible for researchers to establish a link between THM's in drinking water and an increase in human cancer because of the inherent complexities of such epidemiological studies. It did not, however, refute the possible causal relationship between carcinogenesis in humans and the presence of THM's and other carcinogens in water supplies. A study by Cantor and McCabe (99) supported the suspicion that a real link existed between organic contaminants in drinking water supplies and cancer rates in the human population. The study fell short, however, of providing the association. The presence of N-organic compounds in water supplies, from the standpoint of human health, exertion of chlorine demand, reaction with chlorine to form less bactericidal and virucidal chloramines, production of objectionable tastes, and direct carcinogenic and mutagenic effects is therefore significant.

This study also raises concern about the potability of finished water during occurrences of summer blue-green algal blooms. Aside from the taste and odors associated with such occurrences, high levels of N-organic material released by these algae, could result in increased THM formation as well as combined N-chloro forms yielding falsely positive tests for free chlorine. During the occurrence of such algal blooms, superintendents of treatment facilities should be advised to carefully monitor the pH of the water during chlorination and to provide increased organic contaminant removal capabilities in alkaline waters where THM formation is more favored. Removal of organic contaminants could be achieved by use of resins which selectively remove organic contaminants or conventional methods such as carbon adsorption, or chemical coagulation. Because all of the methods currently used to measure free chlorine are subject to interference from organic chloramines, enumeration of bacteria after chlorination should be examined closely in addition to maintaining a chlorine residual somewhat greater than what is normally acceptable, to ensure a sufficiently disinfected water.

SECTION 5

REACTIONS BETWEEN NITROGENOUS ORGANIC COMPOUNDS AND AQUEOUS CHLORINE

A broad spectrum of nitrogenous compounds at concentrations in the range of milligrams per liter were examined for their reactions with aqueous chlorine in dilute aqueous solution. The major types of reactivity studied were: (1) chlorine demand, as a measure of the occurrence of either redox processes or the formation of chlorinated organic compounds; (2) chloramine formation as shown by alteration in the reactions of the residual available chlorine with selective analytical reagents; (3) details of reactions as shown by changes in the ultraviolet spectra of mixtures of the compounds with aqueous chlorine; and (4) analysis of appropriate reaction mixtures for formation of halogenated methanes or other volatile chlorinated compounds resulting from the chlorination of nitrogenous materials.

Selected nitrogenous organic compounds were assessed during a series of screening experiments using chlorine demand and ultraviolet spectrophotometric scans to detect the formation of chlorinated derivatives. The chlorine demand exerted by these compounds was most significant as shown in Table 38. All of these nitrogenous compounds exhibited substantial chlorine demand at concentrations in the range of 1-10 mg per liter within several hours of contact, indicating the importance of nitrogenous materials with respect to the total organic content of natural waters. Although nitrogenous materials may constitute only 5% of the organic matter in a natural water, for example, they may account for 25% of the demand.

The potential of selected compounds as precursors to chloroform formation was also investigated. Initial experiments were carried out near pH 7 with concentrations of aqueous chlorine typically less than 7 milligrams per liter. The amount of chloroform produced as a function of time was determined for selected compounds, at pH values between 6 and 11. Studies were also made to determine the effect of increasing the pH of reaction mixtures after they had been allowed to react at an initial pH value for several hours.

ANALYTICAL METHODS

The concentrations of chlorine solutions and organic reagents typically utilized in the experiments were 10^{-4} molar or less. Frequently, studies were carried out with concentrations as small as one micromole per liter. Because of this, scrupulous care had to be taken to diminish the likelihood of contamination during the preparation of reagents. Furthermore, additional precautions had to be taken throughout the analyses to reduce or eliminate errors due to extraneous substances or specific problems inherent in some of the analytical techniques. Routine laboratory operations were carried out consistently according to the following procedure.

TABLE 38. NITROGENOUS COMPOUNDS UNDER INVESTIGATION

Compound	N-Chlor Formation	Chlorine Demand[a]	U.V. Spectrum Study
Adenine	X	+ 3	X
Alanine		+ 1.5	
m-Aminophenol	X	+ 5	X
Arginine		+ 2.5	
Aspartic Acid		+ 3	
Barbituric Acid	X	+ 4	X
Caffeine	X	0	X
Creatinine	X	+ 1 (v.s.)	X
Cyanuric Acid	X	0	
Cytosine	X	+ 2	X
Glutamic Acid	X	+ 2	
Glycylglycine	X	0	
Histidine	X	+ 2	
Indole	X	+ 8	X
Phenylalanine	X	+ 2.5	X
Proline	X	+ 1.5	
Purine	X	0	
Pyrrole	X	+ 5.5	X
Sarcosine	X	0	
Succinimide	X	0	
Thymine	X	+ 3	
Tryptophan	X	+13	X
Uracil	X	+ 3	X
Uric Acid	X	+ 3	
Tyrosine	X	+ 3	

[a] A plus sign indicates demand greater than one mole of chlorine per mole of compound after several hours of reaction with excess chlorine. Numbers give the moles of chlorine demand per mole of substrate after 5 to 10 hours reaction time with excess chlorine present.

Glassware

Prior to initial use, all glassware was thoroughly cleaned using laboratory detergent, rinsing with dilute HCl, and final rinsing with distilled, chlorine-demand-free water. Spectrophotometric cells were periodically soaked in a solution of 50% ethanol and 50% 3N HCl prior to a distilled water rinse.

Reagent Preparation and Analytical Procedure

Chloride-Free-HOCl Solutions--

In order to minimize the loss of HOCl, concentrated aqueous chlorine stock solutions were prepared under a hood by bubbling chlorine gas from a sintered-glass diffuser into a 2 liter Erlenmeyer flask containing distilled water until a chartreuse color was attained. After neutralization to about pH 6 with concentrated sodium hydroxide, the chlorine solution was distilled in a rotary vacuum distiller at 30-35°C. The condensate (chloride-free) was stored under refrigeration in low-actinic, Pyrex-glass bottles. This concentrated stock solution, which remained stable for months, was used to prepare all aqueous chlorine (HOCl) solutions.

Buffer--To simulate conditions encountered in natural systems, a bicarbonate CO_2 buffer (pH approximately 7.0) was employed. Two methods generally were used to prepare buffer solutions. In the first dilute HCl (and, if necessary, dilute NaOH) was used to adjust the pH of 10^{-3} M (1.0 mM) $NaHCO_3$ solution to 7. In the second method carbon dioxide gas was bubbled through the 10^{-3} M (1.0 mM) $NaHCO_3$ until pH 7 was obtained. With the latter technique, a high background concentration of chloride was avoided.

Phosphate and Borax buffers, shown below, were used to achieve pH values between 5 and 10.

pH	
5.91	KH_2PO_4 (6.00×10^{-4}M); Na_2HPO_4 (8.38×10^{-5}M)
6.98	KH_2PO_4 (2.67×10^{-4}M); Na_2HPO_4 (5.02×10^{-4}M)
7.73	KH_2PO_4 (6.69×10^{-5}M); Na_2HPO_4 (7.53×10^{-4}M)
9.50	$Na_2B_4O_7 \cdot 10\ H_2O$ (.05M)
10	Carbonate/bicarbonate buffer

All buffers were prepared with chlorine-demand-free distilled water and stored in glass carbons.

CHLOROFORM STANDARDS

Standard solutions of chloroform for the calibration of the gas chromatographic equipment were prepared by dissolving 1 ml of chloroform in 20 ml of 9.5% ethanol and diluting to one liter with distilled water. After mixing, 5 ml of this solution was diluted to 250 ml with distilled water, followed by further dilution of 5 ml to one liter with distilled water. This produced a chloroform standard that contained 148 mg per liter of chloroform. Screw cap vaccine vials (25 ml) were filled to overflowing with portions of this final solution, capped with Tuf-Bond (making sure that no air bubbles were formed), and placed in the refrigerator until use. Fresh standards were prepared in this manner at least once a weak.

Equipment

Spectrophotometers--

A Beckman DU spectrophotometer was used for the various colorimetric determinations of free available chlorine. Analyses were carried out in the visible wavelength range with matched one cm cells using distilled water as the reference solution.

A Beckman DK-2 spectrophotometer was used to monitor the ultraviolet absorption of aqueous solutions of individual compounds investigated prior to and during contact with aqueous chlorine. Distilled water was used as the reference solution.

Gas Chromatographs--

Gas chromatographic measurements of concentrations of chloroform produced during experiments were carried out at the Lawrence Experiment Station of the Massachusetts Department of Environmental Quality Engineering in Lawrence, Massachusetts. The instrument most frequently used consisted of a Chromalytics 1047 Concentrator, Tracor 222 gas chromatograph, and Tracor 310 Hall electrolytic conductivity detector. A second chromatograph, the programmable Tracor 560 equipped with a Tekmar Liquid Sample Concentrator (model LSC-1) and a Tracor 700 Hall electrolytic conductivity detector, was used periodically. Although the response of the Tracor 560 was approximately an order of magnitude more sensitive than that of the 222, the selective adsorption principle of operation remained the same. In each case, the volatile components were purged with nitrogen (Matheson cylinder) from a five-ml aqueous sample and collected on a site-sampling tube packed with TENAX resins. The tube was then heated to desorb the adsorbed volatiles and pass them from the first tube to a concentrator U-tube trap again packed with TENAX. Then the latter tube was heated in a programmed manner from 130°F to 180°F to transfer the volatile materials to the chromatographic column. This precolumn technique was needed to provide the slug sample required for gas chromatographic determinations.

Individual compounds were selectively desorbed during the temperature program and combined with deionized solvent in a gas-liquid contactor. The Hall detector continuously monitored the electrical conductivity of the liquid. Thus, the concentrations of the halogenated materials were shown potentiometrically as a strip chart recorder print-out. Peak heights were quantitatively calibrated by comparison with standards which were run at least once each day.

Chlorine-Demand

Chlorine-demand studies were conducted according to the following general procedure: 0.50 - 0.20 millimolar of each organic compound was made up in a 500 ml volumetric flask using 0.01 M $NaHCO_3$ solution. A volume of stock aqueous chlorine solution sufficient to give a molar ratio for chlorine to organic compound usually about 4 or 5 to one was placed in a second 500 ml volumetric flask and diluted to the mark with 0.01 M $NaHCO_3$.

For initiation of reaction the contents of the two flasks were poured simultaneously into a two-liter reagent bottle of low actinic glass. Samples (100 ml) were removed from the reagent bottle at suitable times after mixing and the concentrations of residual chlorine were determined by thiosulfate titration following addition of iodide and acetic acid using starch as indicator. Some attempts were also made to determine free chlorine by titration with methyl orange. The results agreed generally with the total residual chlorine measurements.

Samples from many of the reaction mixtures were placed in spectrophotometric cells and measurements of ultraviolet spectra were made at a number of times with a Beckman-DK2 spectrophotometer. The proportion of chlorine concentration remaining (chlorine at time, t/initial chlorine) was computed at various times.

RESULTS AND DISCUSSION

Chlorine-Demand of N-Organic Compounds

Chlorine-demand provides a direct measure of the overall extent to which organic matter has been oxidized, and, when combined with differential chloride determinations, provides a measure of total chloro-organic compound produced by means of the equation

$$\text{Cl-demand} = \text{Cl}^{-}\text{-formed} + \text{C-Cl bonds formed} \quad . \qquad (13)$$

Studies concerning the chlorine demand of selected N-organic compounds are shown graphically in Figures 110 through 121.

The chlorine-demand ratio (CDR) is given by equation (14):

$$\text{Chlorine-demand ratio (CDR)} = \frac{(\text{Cl}_o) - (\text{Cl}_t)}{(\text{Cpd}_o)} \qquad (14)$$

where

Cl_o = the added initial molar concentration of chlorine in the reaction mixture

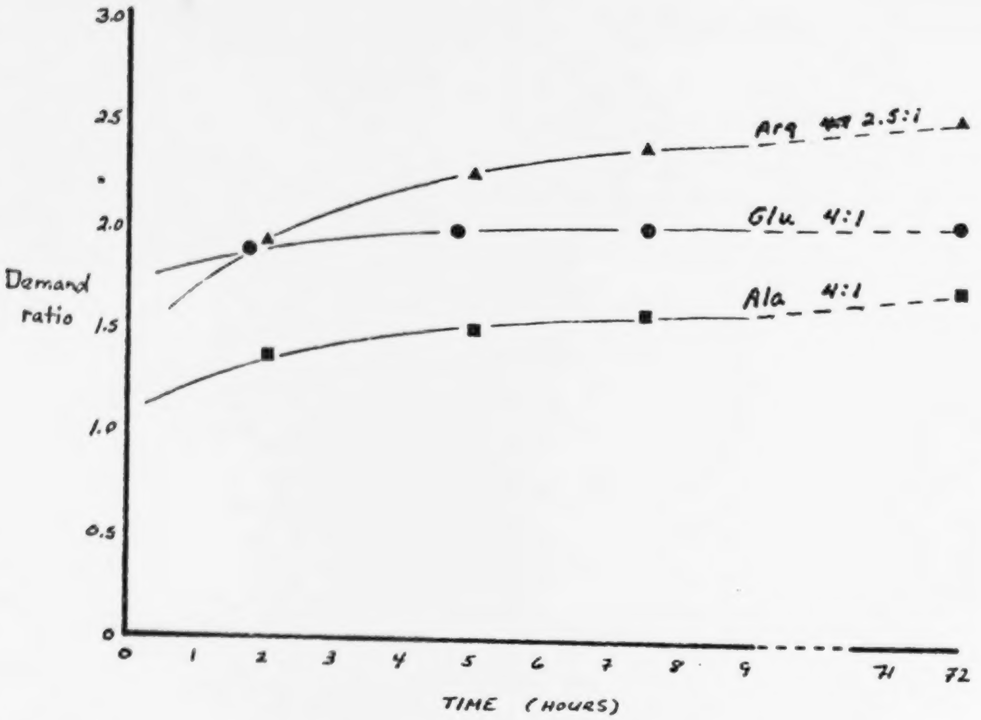
Cl_t = the molar concentration of chlorine remaining at any time, t

Cpd_o = the added initial molar concentration of compound in the reaction mixture.

Alanine, $CH_3CH(NH_2)COOH$--

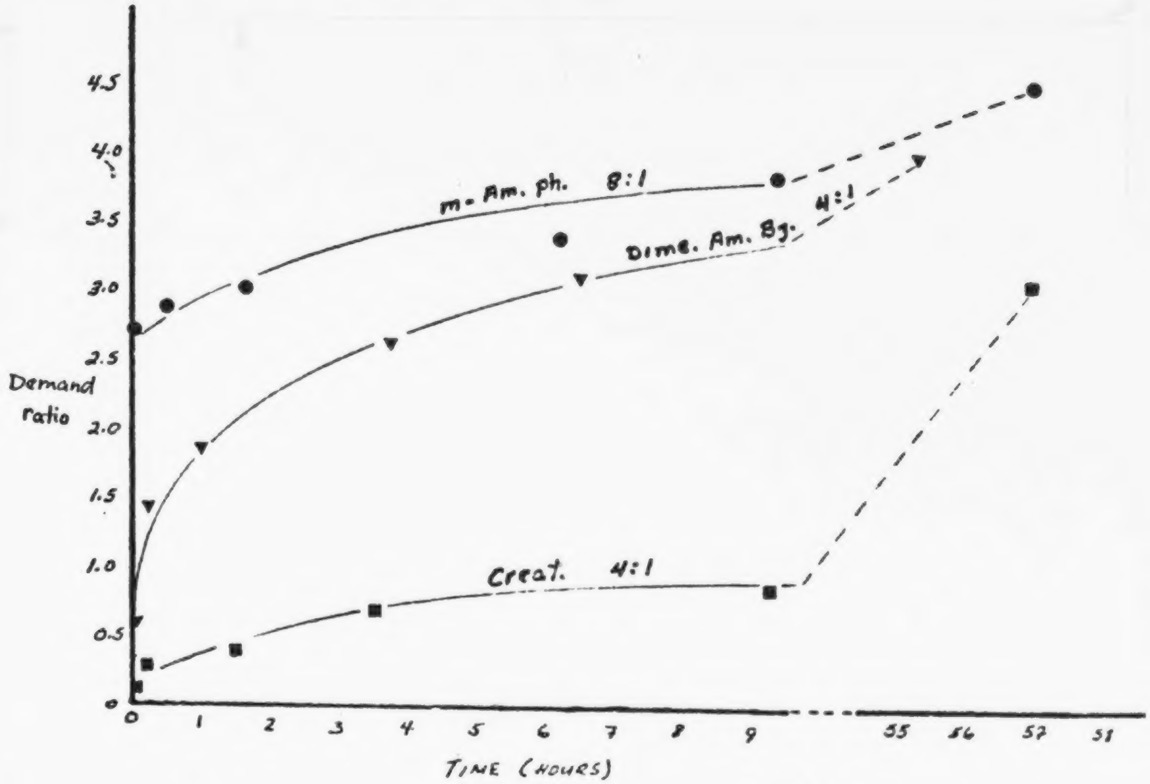
A demand experiment was made on this typical amino acid at a molar ratio of aqueous chlorine to alanine equal to 4, the initial concentrations being 5×10^{-5} alanine, and 2×10^{-4}M chlorine. The results obtained were:

time, hr.	2	5	7.5	72
demand ratio	1.4	1.5	1.6	1.7



		mM	ppm	molar ratio	
—●—	Glutamic acid	.05	7.36	4:1	$HOOCCH_2CH_2CH(NH_2)COOH$
	Chlorine	.20	14.15		
—■—	L-Alanine	.05	4.45	4:1	$CH_3CH(NH_2)COOH$
	Chlorine	.20	14.15		
—▲—	Arginine	.05	8.71	4:1	$C_6H_{14}N_4O_2$
	Chlorine	.02	14.15		

Figure 110. Chlorine demand of selected N-organic compounds.



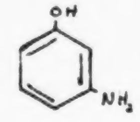
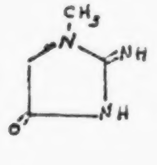
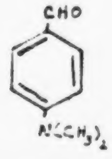
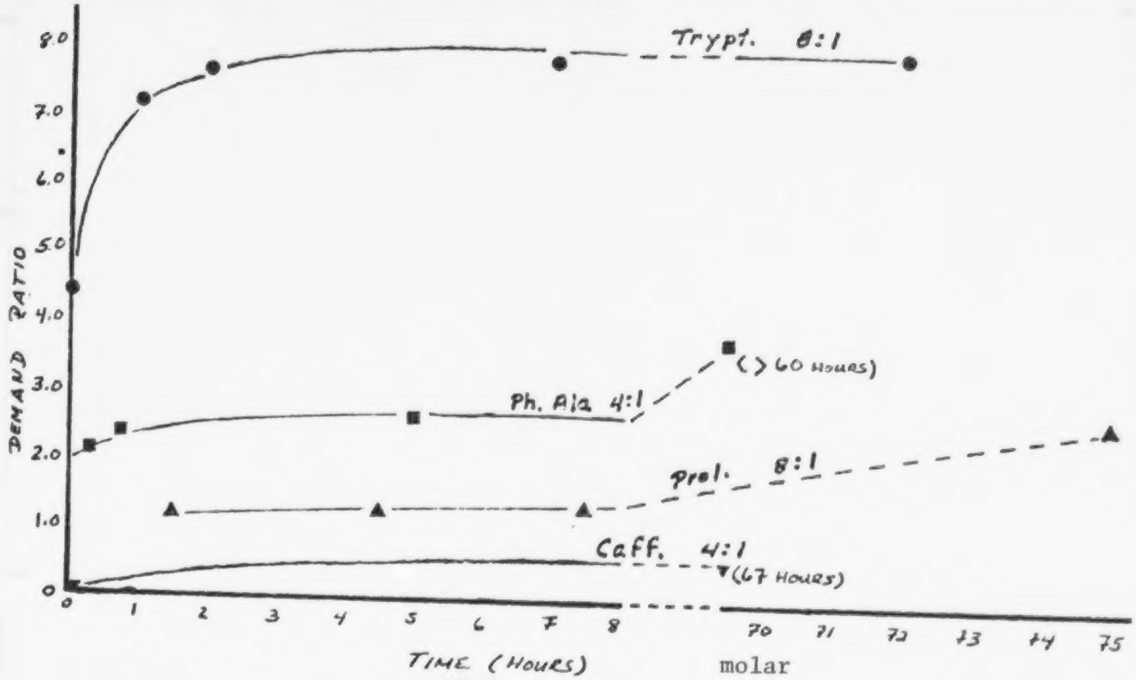
		mM	ppm	molar ratio	
	m-Aminophenol	.10	10.9		
—●—				8:1	
	Chlorine	.80	56.6		
	Creatinine	.05	5.65		
—■—				4:1	
	Chlorine	.20	14.15		
	Dimethylamino-benzaldehyde	.10	14.9		
—▼—				4:1	
	Chlorine	.40	28.3		

Figure 111. Chlorine demand of selected N-organic compounds.



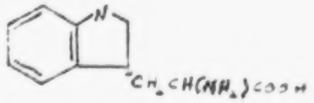
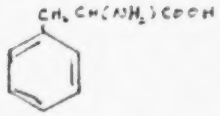
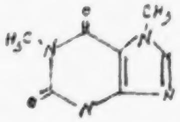
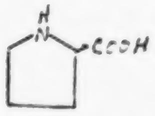
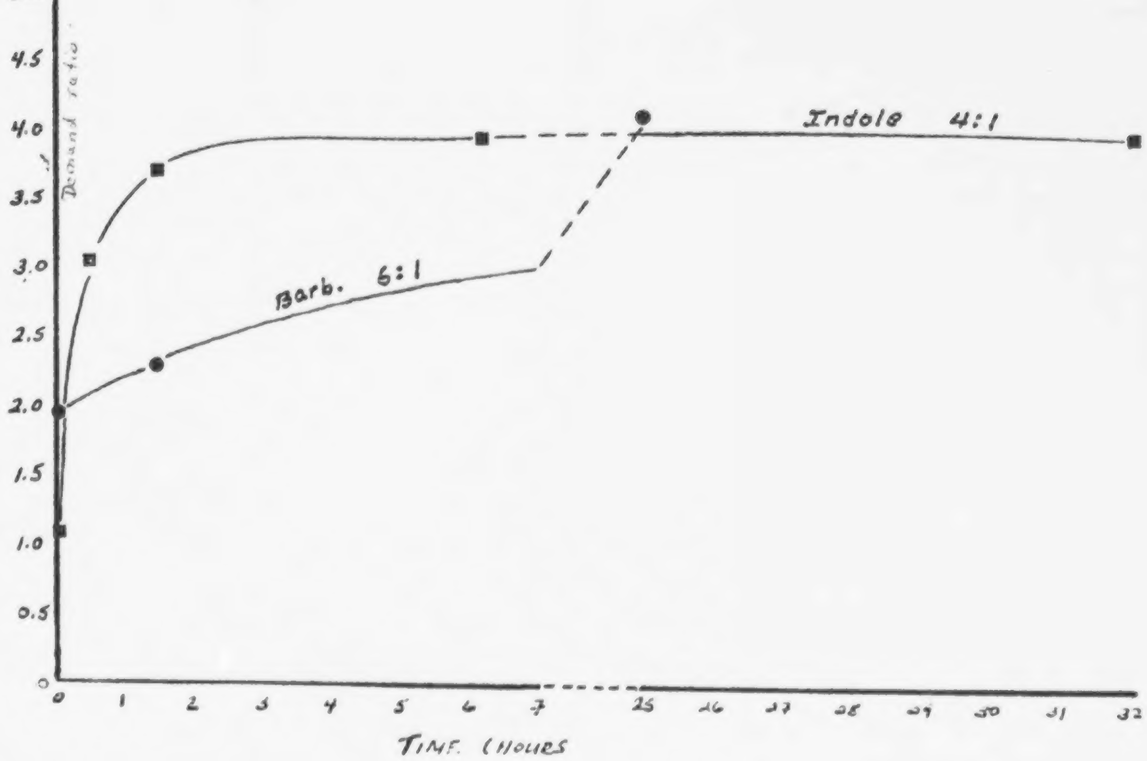
	mM	ppm	molar ratio	
L-Tryptophan	.05	10.2	8:1	 N CH2CH(NH2)COOH
—●—				
Chlorine	.20	14.15		
Phenylalanine	1.4	231.0	4:1	 CH2CH(NH2)COOH
—■—				
Chlorine	5.6	396.0		
Caffeine	.05	9.7	4:1	 O CH3 H3C N N N N O
—▼—				
Chlorine	.20	14.15		
Proline	.05	5.76	8:1	
—▲—				
Chlorine	.40	28.30		

Figure 112. Chlorine demand of selected N-organic compounds.



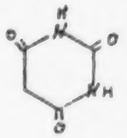
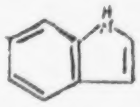
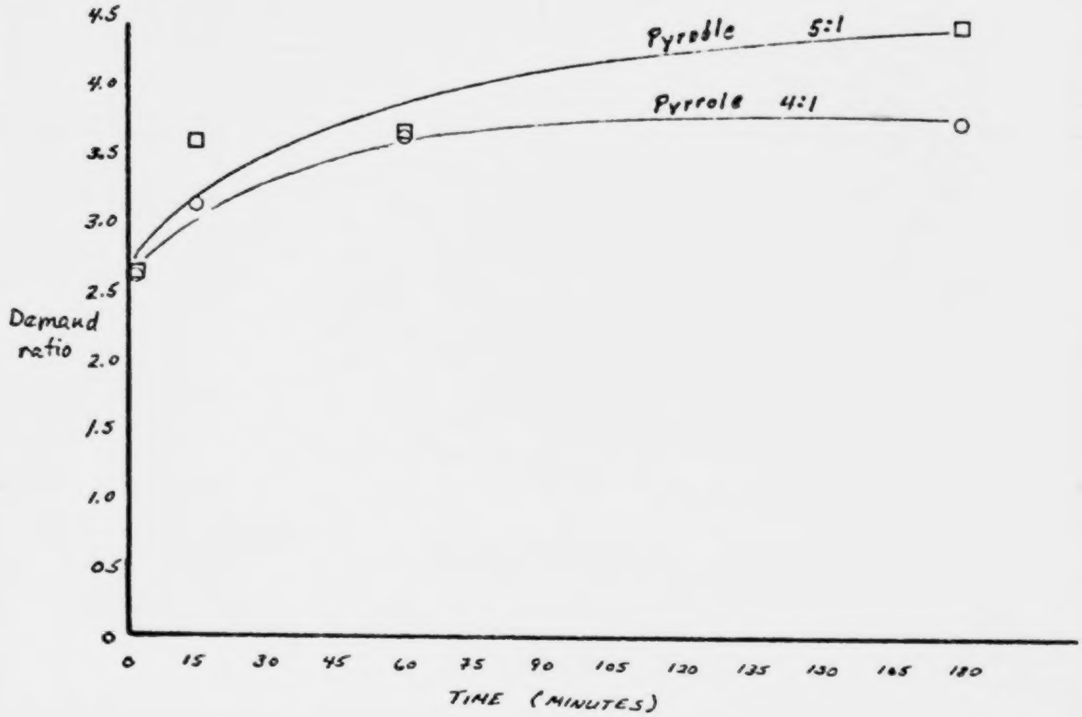
	mM	ppm	molar ratio	
Barbituric acid	.10	12.8		
—●—			6:1	
Chlorine	.60	42.5		
Indole	.05	5.86		
—■—			4:1	
Chlorine	.20	14.15		

Figure 113. Chlorine demand of selected N-organic compounds.



		mM	ppm	molar ratio
—○—	Pyrrole	.15	10.1	4:1
	Chlorine	.60	42.5	
—□—	Pyrrole	.30	20.2	5:1
	Chlorine	1.50	106.2	

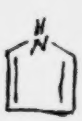
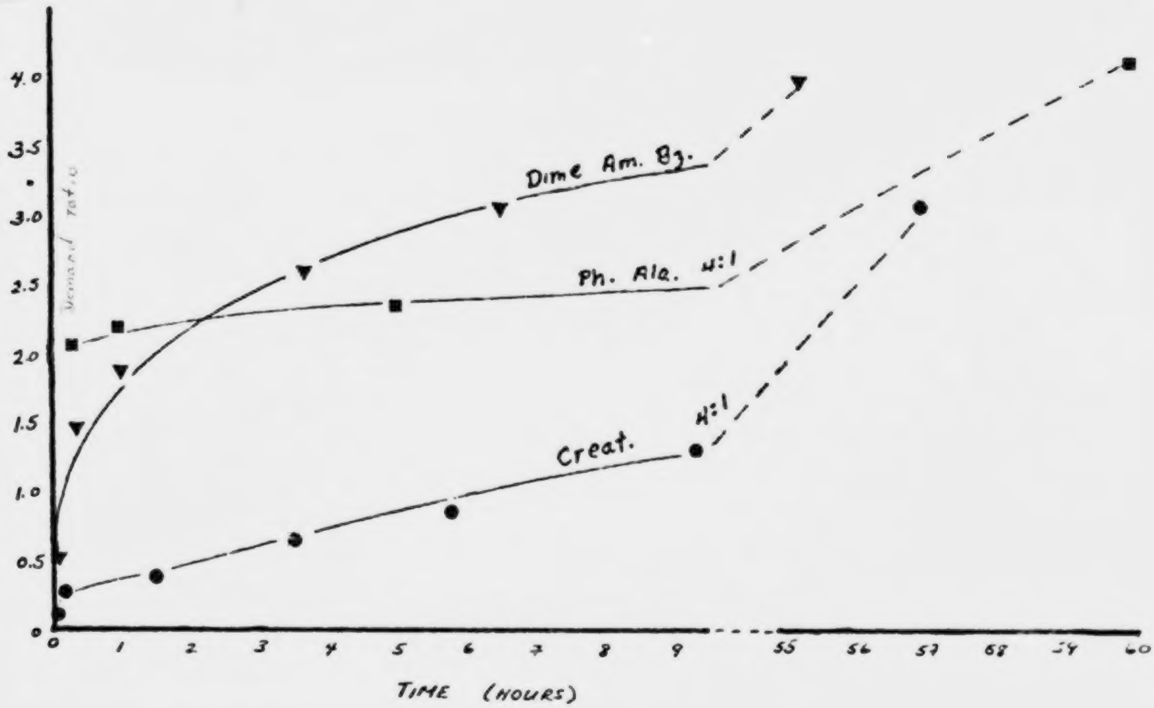
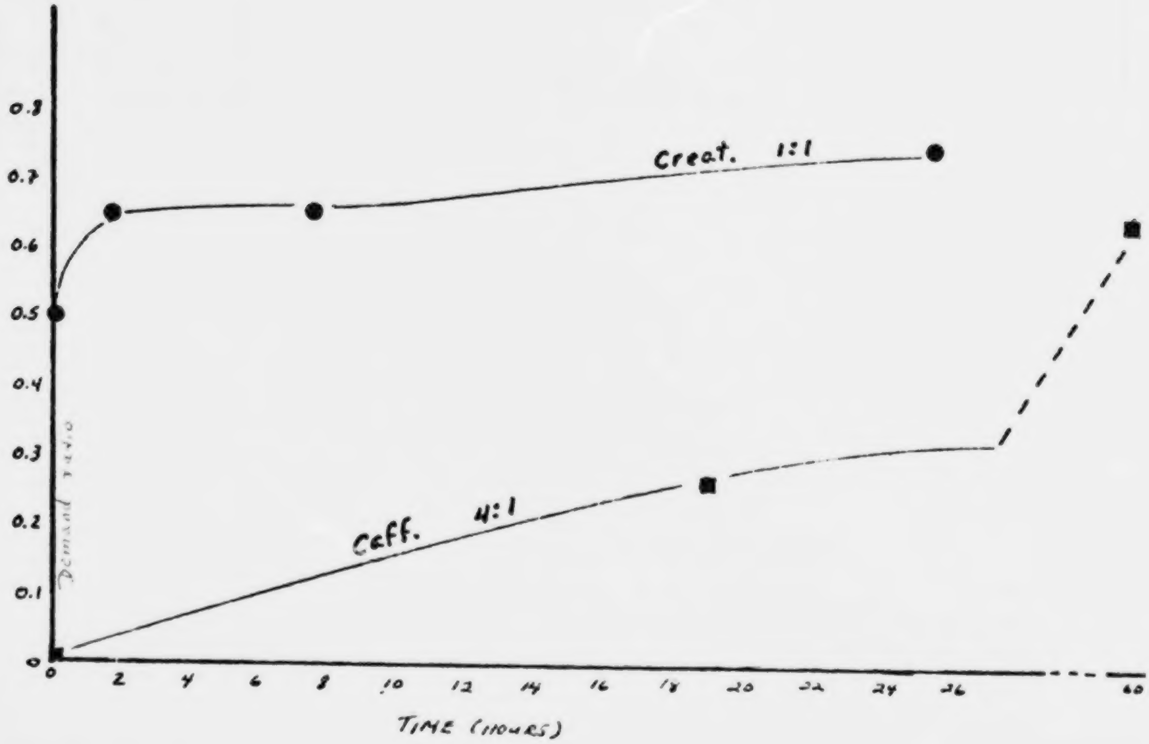


Figure 114. Chlorine demand of selected N-organic compounds.



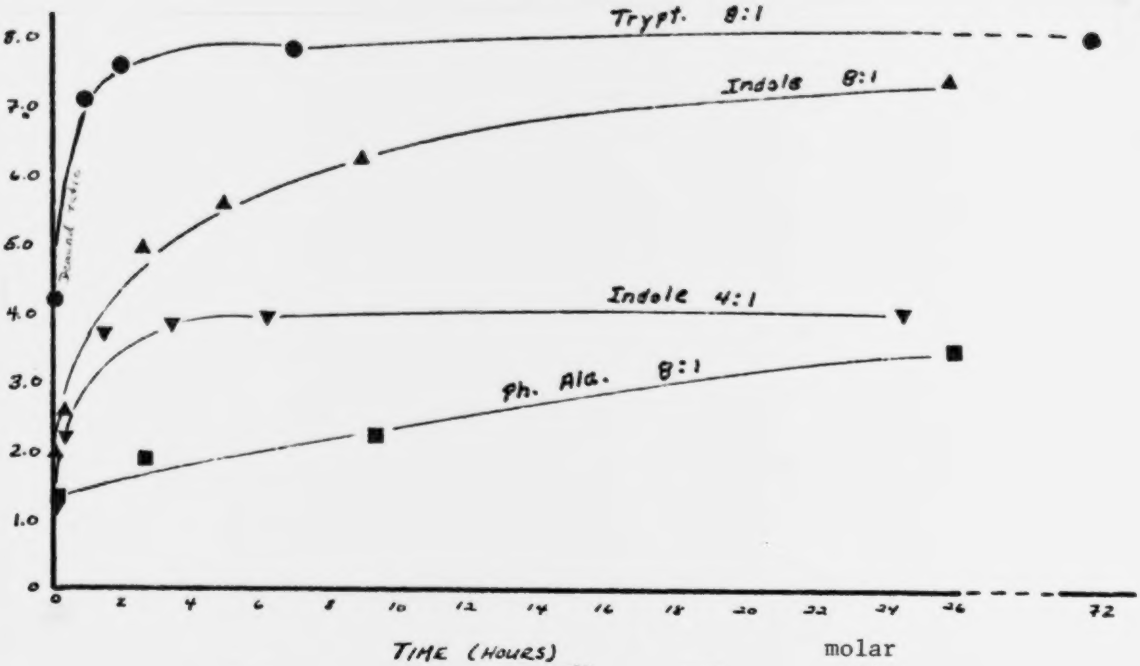
	mM	ppm	molar ratio
Creatinine	.05	5.65	
—●—			4:1
Chlorine	.20	14.15	
Phenylalanine	1.4	231.0	
—■—			4:1
Chlorine	5.6	396.0	
Dimethylamino-benzaldehyde	.10	14.9	
—▼—			4:1
Chlorine	.40	28.30	

Figure 115. Chlorine demand of selected N-organic compounds.



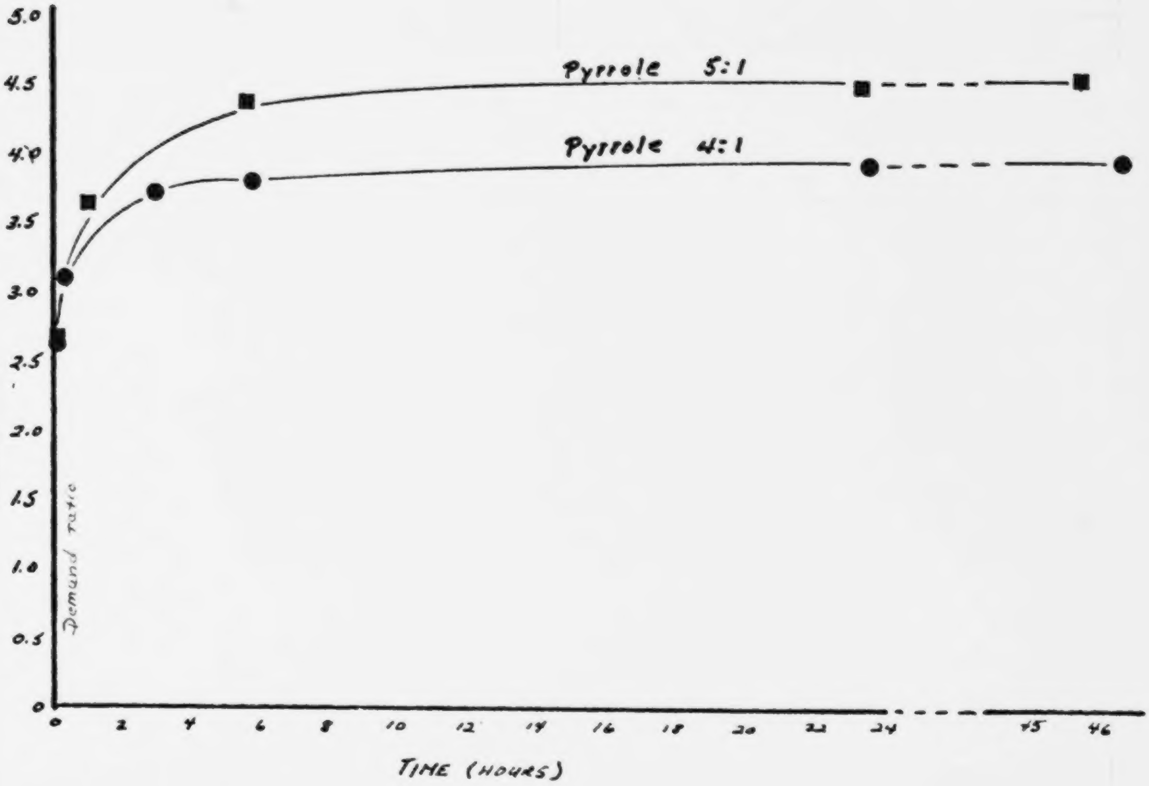
	mM	ppm	molar ratio
Creatinine	.10	10.3	
— ● —			1:1
Chlorine	.10	7.08	
Caffeine	.05	9.7	
— ■ —			4:1
Chlorine	.20	14.15	

Figure 116. Chlorine demand of selected N-organic compounds.



		mM	ppm	molar ratio
—●—	L-Tryptophan	.05	10.2	8:1
	Chlorine	.40	28.3	
—■—	Phenylalanine	.50	82.5	8:1
	Chlorine	4.00	283.0	
—▲—	Indole	.05	5.85	8:1
	Chlorine	.40	28.3	
—▼—	Indole	.05	5.86	4:1
	Chlorine	.20	14.15	

Figure 117. Chlorine demand of selected N-organic compounds.



		mM	ppm	molar ratio
— ■ —	Pyrrole	.30	20.1	5:1
	Chlorine	1.50	106.25	
— ● —	Pyrrole	.15	10.1	4:1
	Chlorine	.60	42.5	

Figure 118. Chlorine demand of selected N-organic compounds.

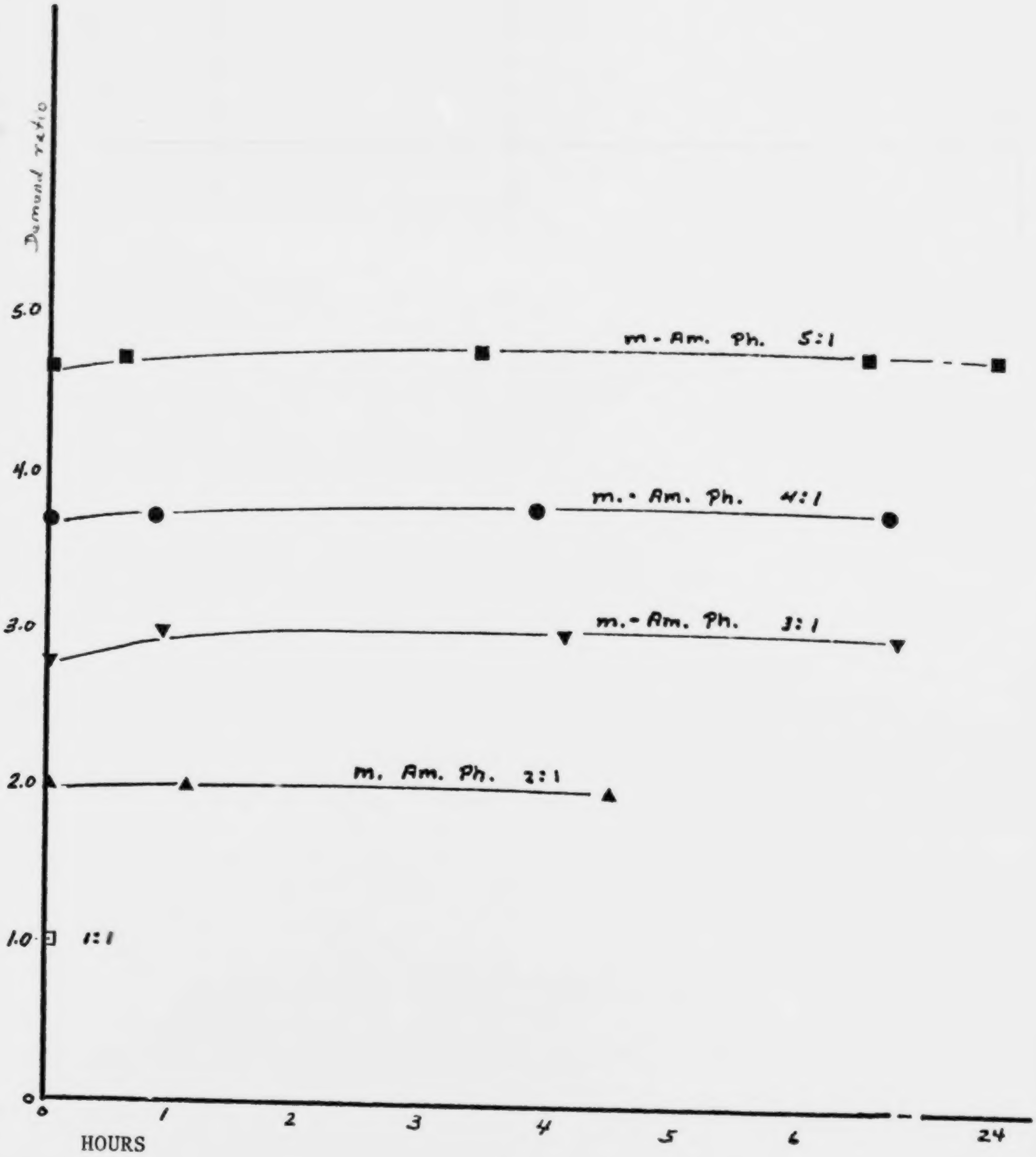
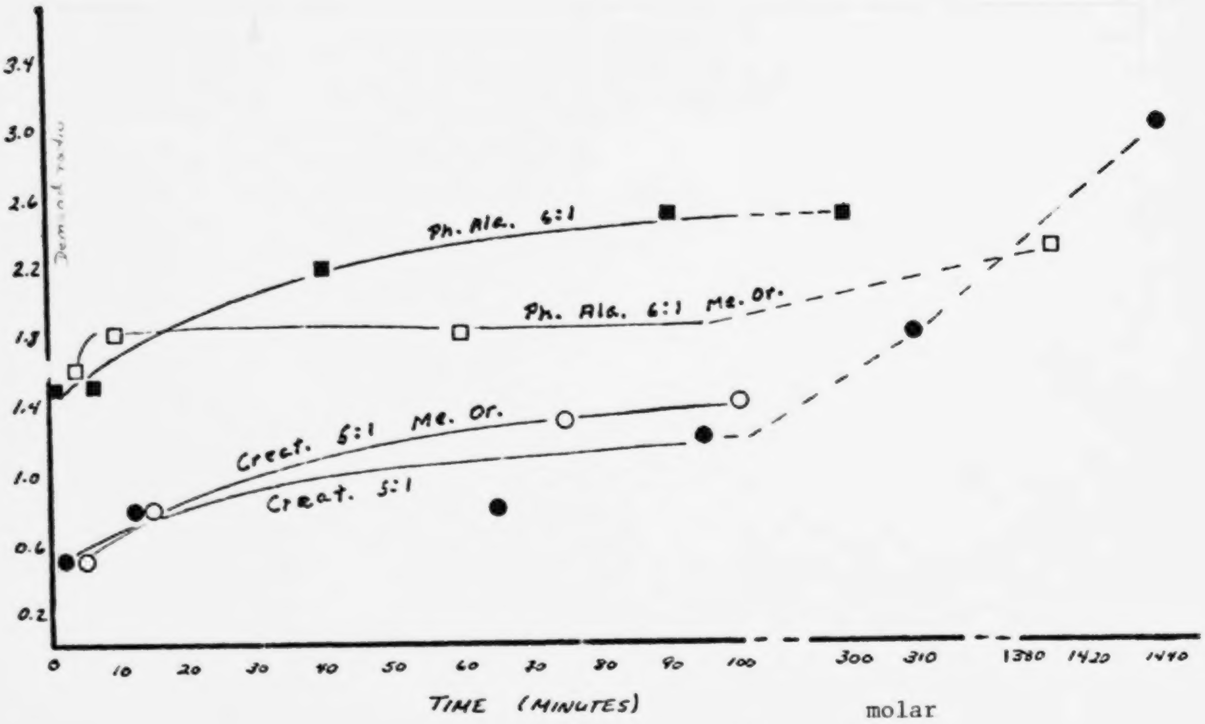


Figure 119. Chlorine demand of selected N-organic compounds.

		mM	ppm	molar ratio
—■—	m-Aminophenol	.10	10.9	5:1
	Chlorine	.50	35.4	
—●—	m-Aminophenol	.10	10.9	4:1
	Chlorine	.40	28.3	
—▼—	m-Aminophenol	.10	10.9	3:1
	Chlorine	.30	21.24	
—▲—	m-Aminophenol	.10	10.9	2:1
	Chlorine	.20	14.15	
—□—	m-Aminophenol	.10	10.9	1:1
	Chlorine	.10	7.08	

Figure 119 (continued)



	MM	ppm	molar ratio
Phenylanine	.01	1.65	
—■—			6:1
Chlorine	.06	4.25	

—□- same as above, determined by Methyl Orange method

Creatinine	.01	1.13	
—●—			5:1
Chlorine	.05	3.54	

—○-same as above, determined by Methyl Orange method

Figure 120. Chlorine demand of selected N-organic compounds.

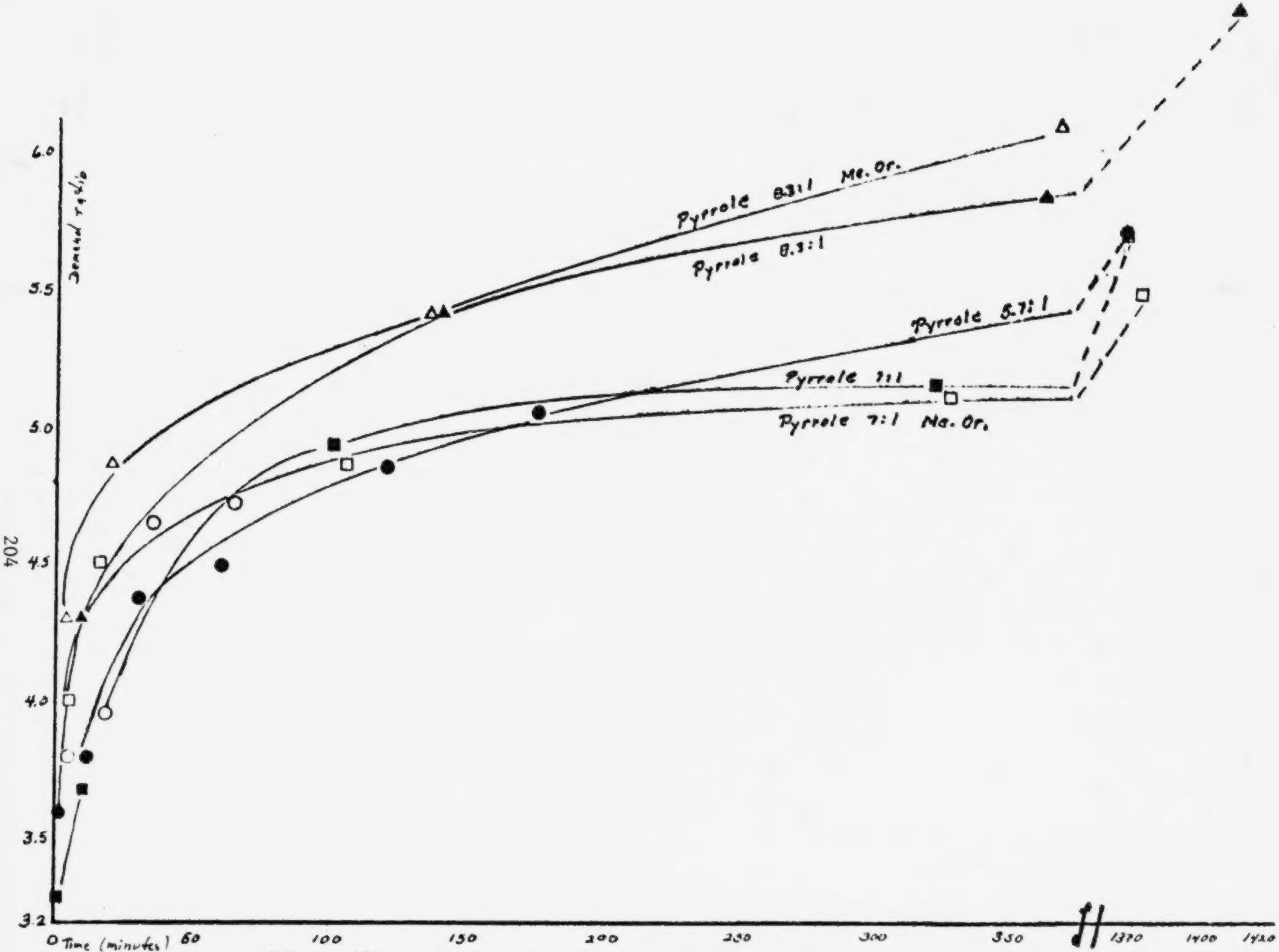


Figure 121. Chlorine demand of selected N-organic compounds.

		mM	ppm	molar ratio
—▲—	Pyrrole	.0072	.48	6.3:1
	Chlorine	.06	4.25	

—△-same as above, determined by Methyl Orange method

—■—	Pyrrole	.0144	.97	7:1
	Chlorine	.10	7.08	

—□-same as above, determined by Methyl Orange method

—●—	Pyrrole	.0144	.97	5.7:1
	Chlorine	.08	5.66	

—○-same as above, as determined by Methyl Orange method

Figure 121 (continued)

(Demands, here and in the other tables, are expressed as moles of chlorine reduced per initial mole of organic compound.)

The results are reasonably consistent with the equation;

$$CH_3CH(NH_2)COOH + 2HOC\ell \rightarrow CH_3COCOOH + NH_2C\ell + H^+ + C\ell^- + H_2O$$

provided there is some concurrent or subsequent oxidation of ammonia to nitrogen.

Arginine, $H_2NCH_2CH_2CH(NH_2)COOH$--

This basic amino acid is of interest because of the alternate opportunities for N-chlor compound formation. An experiment with a molar ratio of aqueous chlorine to arginine equal to 4, the initial concentrations being 5×10^{-5}M arginine and 2×10^{-2}M chlorine, gave the following data:

time, hr.	2	5	7.5	72
demand	1.9	2.3	2.4	2.5

The results suggest oxidative deamination at one amino group followed by breakpoint oxidation of the liberated ammonia. The fact that the behavior is similar to that of other natural amino acids suggests that this reaction is occurring at the α-amino group. The suggested reaction, then, is

$$H_2NCH_2CH_2CH(NH_2)COOH + 2.5\ HOC\ell \rightarrow H_2NCH_2CH_2COCOOH + \frac{1}{2} N_2 + 2.5\ C\ell^- + 2.5\ H^+ + 1.5\ H_2O\ .$$

Creatinine, $C_4H_7N_3O$--

Creatinine, a urinary excretory product, was considered a likely nitrogenous constituent of waters polluted by animals or man. It has two prospective sites for N-chlorination, one a ring N-H, amide-like in structure, the other an amino group.

Three kinetic runs were performed with mixtures of creatinine and aqueous chlorine, at molar chlorine to creatinine ratios of 1:1, 4:1 and 5:1.

Mixture 1: 1×10^{-4}M chlorine; 1×10^{-4}M creatinine

time, hr.	0.1	2	7.9	25.5
demand	0.5	0.65	0.65	0.75

Mixture 2: 2×10^{-4}M chlorine; 5×10^{-5}M creatinine

time, hr.	0.1	0.25	1.5	3.5	5.8	9.3	57
demand	0.1	0.3	0.4	0.7	0.85	1.3	3.1

Mixture 3: 5×10^{-5}M chlorine; 1×10^{-5}M creatinine

time, hr.	0.1	0.25	1.2	1.6	5	24
demand	0.5	0.8	1.0	1.3	1.8	3.0

Concurrent spectrophotometric observations on the 1:1 and 4:1 mixtures were not very informative. The 1:1 mixture showed enhanced absorbance in the region near 230 nm, the wavelength of maximum absorption for creatine. This might indicate of an N-chloro derivative or oxidation product of creatinine in which the fundamental absorbing structure had not been changed. The absorption at 230 nm continued increasing throughout the run for this chlorine to creatinine ratio. With the 4:1 mixture the increase was less pronounced, the wavelength of maximum absorption was shifted toward 220 nm and the overall absorption began decreasing after about 9 hours of reaction and continued to do so subsequently throughout the reaction period.

Creatinine thus exhibits a small immediate demand followed by a continued slow exertion of demand to at least 3 moles of chlorine for each mole of creatinine. Presumably some oxidized intermediate is formed which is then subject to additional oxidation slowly in the presence of excess chlorine. Other than this, no plausible reaction scheme can be proposed at present.

Glutamic Acid, $HOOC_2CH_2CH\ CH(NH_2)COOH$--

A single experiment at 4:1 molar ratio of chlorine to glutamic acid, the concentrations being 2×10^{-4}M and 5×10^{-5}M, gave the following results:

time, hr.	2	5	7.5	72
demand	1.8	2.0	2.0	2.0

Although the molar demand is a bit low for completion of the reaction

$$HOOCCH_2CH_2CH(NH_2)COOH + 2.5\ HOC\ell \rightarrow HOOCCH_2CH_2COCOOH + 2.5\ C\ell^- + 0.5\ N_2 + 2.5\ H^+ + 1.5\ H_2) \quad ,$$

this seems the most reasonable mechanism to propose. A corresponding experiment with aspartic acid, $HOOCCH_2CH(NH_2)COOH$, gave somewhat greater molar demands, 1.8 after 2 hours, 2.0 after 5 hours and 3.5 at 72 hours. There is potential chloroform formation from the initial oxaloacetic acid product, $HOOCCH_2COCOOH$ in this case.

Indole, C_8H_7N--

Indole is one of the substances studied showing strong chlorine demand. At chlorine to indole ratios up to 4 the chlorine was essentially completely reduced in an hour or less. The tabulated data are for molar ratios of 4:1 and 8:1, both with 5×10^{-5}M indole, the initial chlorine concentrations being 2×10^{-4}M and 4×10^{-4}M, respectively.

4:1 molar ratio

time, hrs.	0.1	0.5	1.5	3.5	6.2	25	32
demand	1.1	2.6	3.7	3.9	4.0	4.0	4.0

8:1 molar ratio

time, hrs.	0.1	0.5	2.6	5	9	26
demand	2.0	2.5	4.9	5.5	6.2	7.4

Although the reaction of chlorine in these experiments was extensive, the maximum chlorine demand shown, 7.4 moles of chlorine per mole of indole, is still far less than the 19.5 moles needed for complete oxidation to CO_2, N_2 and H_2O. Accordingly, there is good indication of partially oxidized organic intermediates, but whether these are chlorinated or not cannot be judged.

Formation of partial oxidation products was also shown visually by the appearance of yellow colors in both solutions within 0.5 hr. of the start of reaction that persisted throughout the runs to at least 55 hours with the 4:1 molar ratio.

Spectrophotometrically, both reaction mixtures exhibited initial intensification of absorption in the 270-280 nm absorption band and a batho chromic shift of absorption near 220 nm. After 30 minues these maxima gradually diminished and were replaced by increasing absorption near 250 nm. This absorption continued to increase for 24 hours in the solution with the molar ratio equal to 4 and then became substantially constant. At the molar ratio of 8, maximum absorption at 250 nm was reached after 80 minutes; thereafter the absorption decreased continuously throughout the ultraviolet range, indicating general oxidation to nonabsorbing products.

m-Aminophenol, C_6H_7ON--

This compound is of interest because of its structural similarity to resorcinol, which Rook (35) found to yield molar quantities of chloroform when chlorinated. It also possesses structural similarities to some naturally occurring aromatic amines. Because ingested amines may react in the stomach with nitrite from saliva to give nitrosamines, the general destructiveness of aqueous chlorine toward amines with consequent elimination of carcinogenic potential is noteworthy as a contrast to the general nonreactivity of chlorine dioxide and ozone towards amines and amides as a class.

A series of experiments was conducted with aqueous chlorine and *m*-aminophenol at molar chlorine to amine ratios ranging from 0.5 to 8. Substantially complete disappearance of available chlorine occurred within two minutes for all but the two largest ratios, 5 and 8. Demand data for the runs at these two ratios, both with 1.0×10^{-4}M *m*-aminophenol, were as follows:

5:1 molar ratio; initial Cl = 5×10^{-4}M

time, hr.	0.1	0.6	3.5	7	24
demand	4.6	4.7	4.8	4.8	4.8

8:1 molar ratio; initial Cl = 8×10^{-4}M

time, hr.	0.1	0.5	1.7	6.3	9.5	57
demand	2.7	2.9	3.0	3.4	3.8	4.5

The reason for the relatively small molar demand observed with the 8:1 chlorine to amine ratio is unknown. It is inconsistent with the rapidity and completeness of chlorine consumption observed in all the other experiments.

A number of color changes was observed in the different reaction mixtures. With the 0.5 molar ratio of chlorine to amine a pale pink color developed as the reactants were mixed. This color persisted for some time; the solution changed to a peach hue over several hours and eventually became pale yellow after about 30 hours. With molar chlorine to amine ratios of 1, 2 and 3 the solutions developed more intense pink colors that persisted for longer times. In all instances, however, the colors had changed to peach after about 18 hours and faded to haylike tinges after 25 to 30 hours. At the molar chlorine to amine ratio of 5 there were flashes of pink color during mixing, but the fully mixed solution was initially pale yellow and became colorless within 3 minutes. Formation of transient intermediates that are further oxidized in the presence of excess free chlorine is indicated.

Ultraviolet spectral changes during these color shifts were not dramatic. Generally increased ultraviolet absorbance was observed for all studied ratios of chlorine to amine. The increase was quite large for the first few minutes of reaction, but rather minor thereafter. An absorption band developed near 310 nm, most prominently when the chlorine to amine ratio was 3.

Phenylalanine, $C_8H_{11}O_2N$

Three experiments were conducted with this amino acid, at molar chlorine to amine ratios of 4, 6 and 8. The results obtained were:

4:1 ratio; 5.6×10^{-3}M Cl, 1.4×10^{-3}M phenylalanine

time, hr.	0.2	0.8	5	60
demand	2.1	2.4	2.6	3.8

6:1 ratio; 6×10^{-5}M Cl, 1×10^{-5}M phenylalanine

time, hr.	0.1	0.2	0.7	1.5	5
demand	1.6	1.7	2.2	2.5	2.5

8:1 ratio; 4×10^{-3}M Cl, 5×10^{-4}M phenylalanine

time, hr.	0.1	2.8	9.4	26
demand	1.4	1.9	2.3	3.5

The observed demands, for the first 5 hours or so, corresponded to the expected value of 2.5 for the reaction.

$$C_6H_5CH_2CH(NH_2)COOH + 2.5\ HOC\ell \rightarrow C_6H_5CH_2COCOOH + \frac{1}{2} N_2 + \frac{3}{2} H_2O + \frac{5}{2} C\ell^- + \frac{5}{2} H^+ \quad .$$

A pungent odor, characterized as sharp, cabbage-like and "organic", was produced during the reaction between aqueous chlorine and phenylalanine. It may be due to phenylpyruvic acid or perhaps to some chlorinated intermediates. Its occurrence suggests a source of tastes and odors developed as a result of chlorination.

Spectrophotometric observations showed a strong increase of overall absorption in the region of the 257 nm peak of phenylalanine soon after start of reaction, but no loss of structure of the absorption band occurred, indicating lack of change in the benzene rings itself. At the molar ratio of chlorine to amino acid equal to 4 the spectrum became quite stable after about 30 minutes. At the molar ratio of 8 there was a continuing slow increase in absorption at 250-260 nm up to 26 hours.

Pyrrole, C_4H_4N--

Several quantitative demand runs were conducted with pyrrole at molar chlorine to amine ratios ranging from 4 to 8.3. Qualitative observations were made on mixtures with smaller ratios.

When pyrrole was mixed at the 10^{-4} level with an equal molarity of aqueous chlorine, there was essentially complete reaction of the available chlorine within 2 minutes. The reaction was accompanied by increased ultraviolet absorption, indicating that the process was not simple oxidative destruction of the pyrrole. Little further spectral change occurred for the next several hours, but overnight a small absorption maximum appeared at 280 nm, indicating continuing reaction even in the absence of available chlorine.

Data from the quantitative runs were as follows:

ratio 4:1; 6×10^{-4} M Cℓ, 1.5×10^{-4} M pyrrole

time, hr.	0.1	0.25	1.0	3.0	6.0	23.5	46
demand	2.6	3.1	3.6	3.7	3.8	3.9	3.9

ratio 5:1; 1.5×10^{-3} M Cℓ, 3×10^{-4} M pyrrole

time, hr.	0.1	0.25	1.0	3.0	6.0	23	46
demand	2.6	3.6	3.7	4.2	4.4	4.5	4.6

ratio 5.7:1; 8×10^{-5} M Cℓ, 1.4×10^{-5} M pyrrole

time, hr.	0.1	0.2	0.5	1.0	2.0	3.0	23
demand	3.7	3.9	4.4	4.5	4.8	5.0	5.7

ratio 7:1; 1×10^{-4} M Cℓ, 1.4×10^{-5} M pyrrole

time, hr.	0.1	0.2	1.8	5.3	23
demand	3.3	3.7	4.9	5.1	5.7

ratio 8.3:1; 6×10^{-5} M Cℓ, 0.72×10^{-5} M pyrrole

time, hr.	0.1	0.3	2.3	5.2	24
demand	4.3	4.8	5.4	5.8	6.5

As can be seen, the molar demand of pyrrole for aqueous chlorine is great, increasing to at least 6.5 when there is substantial residual chlorine in the solution. When the initial molar ratio of pyrrole to chlorine was 4, the nearly immediate molar demand of about 3 was accompanied by the development of an ultraviolet absorption band with a maximum near 250 nm. The intensity of this band decreased gradually over the succeeding several hours as the remaining available chlorine reacted. Similar results were obtained with an initial molar ratio of 5 except that the demand seemed to stabilize at about 4.5 with some residual chlorine left in the solution after about six hours reaction. However, continuing greater demand was shown with greater molar ratios of chlorine to pyrrole, showing that additional oxidation was still possible.

The reactivity of pyrrole with aqueous chlorine was particularly important because of the relation of pyrrole to porphyrins (such as chlorophyll and heme), the essential amino acid tryptophane, and other decomposition products of proteins (such as indole and indole derivatives). Tryptophane and indole, both of which contain pyrrole rings, exerted significant chlorine demands. A third nitrogen heterocycle, proline, having a pyrrolidine structure, also exhibited a high chlorine demand.

The results so far point to considerable destructive oxidation of pyrrole by aqueous chlorine that overshadows possible chloramine formation. The spectra show the formation of absorptive intermediates, some of which may be chlorinated. It seems likely that pyrrole may be a source of volatile chlorinated organic compounds like chloroform.

Tryptophane, $C_{11}H_{12}O_2N_2$--

Tryptophane is an amino acid of interest because it contains both a pyrrolic-N and a primary amino-N. The pyrrole function appears to take precedence, for the immediate molar chlorine demand of tryptophane solutions is greater than 3 and significant residual chlorine does not last for much more than 2 hours even at an initial molar ratio of chlorine to tryptophane equal to 8. There is some indication that the demand for 10^{-5}M solutions over 24 hours may be approximately 12.

Quantitative results for the initial molar ratio equal to 8, with 4×10^{-4}M chlorine and 5×10^{-5}M tryptophane, were:

time, hr.	0.1	1.0	2.0	7	7.2
demand	4.2	7.1	7.6	7.8	8.0

Spectrophotometric observations on the reaction mixture with the 8:1 molar ratio indicated a complex reaction picture. Initially the tryptophane absorption peak near 280 nm was replaced by a stronger absorption band with maximum near 265 nm. Then this band was superseded over a period of about 30 minutes by another with a maximum near 240 nm. The intensity of this last absorption continued increasing for at least 5 hours and a small secondary peak with maximum near 330 nm also developed. A visible yellow color also appeared in the solution after about 2 hours.

It appears probable that there is extensive reaction with the pyrrolic ring at least and that volatile chlorinated products may be formed as with pyrrole itself.

Barbituric Acid--

The reaction of aqueous chlorine with barbituric acid was studied with a 6:1 molar ratio of chlorine to barbiturate at 10^{-4}M concentration.

There was a rapid initial demand of 2.0 moles of chlorine per mole of babiturate that was accompanied by a substantially complete disappearance of the barbiturate absorption maximum at 250-260 nm. Substituted for the absorption maximum was a generally increasing absorption toward shorter wave lengths. This general absorption then decreased slowly with increasing reaction time. The results imply a rapid partial reaction with all the barbiturate rather than a more extensive reaction with some of it.

The molar chlorine demand increased slowly with increasing time of reaction from 2.8 to 75 minutes to 4.3 at 27 hours.

Caffeine--

This purine differs from the barbiturate in that the pyrimidine nitrogens are fully methylated and there are no methylene hydrogens alpha to carbonyl groups as potential chlorination sites. As a result very little reaction with chlorine was observed. In a mixture of 2×10^{-4}M chlorine with $.5 \times 10^{-4}$M caffeine the chlorine decreased only to 1.7×10^{-4} over a period of 67 hours. Correspondingly the absorption spectrum for caffeine with its maximum at 273 nm changed only slightly. There was a slight general decrease in absorption over the 67 hour period, perhaps corresponding to the slight reduction observed in the chlorine concentration.

p-Dymethylaminobenzaldehyde--

This substance, like the previous one, is not of direct, primary concern. It happened to be available as a representative of a para-substituted aromatic derivative and a structural analog of p-aminobenzoate.

In the one run with this compound, at a molar ratio of 4, there was an initial molar demand about 0.6 that increased to 1.5 over 10 minutes, to about 2.0 after 2 hours, to 3.0 after 6 hours and eventually to 4.0 after 55 hours.

Spectrophotometrically there was an initial shift in absorption from a peak at 340 nm to one at 315 nm and from one at 245 nm to a broad maximum centered near 233 nm that was complete in 10 minutes. Subsequently, the absorption remained substantially constant for about 4 hours followed by a slow uniform decrease in intensity across the spectrum for the duration of the experiment.

Cytosine--

One series of studies was carried out in which fairly concentrated solutions of cytosine were mixed with portions of aqueous chlorine, the molar ratios of chlorine to cytosine ranging from less than one to several fold. After overnight reaction the solutions were evaporated under vacuum and the concentrated residues were subjected to thin-layer chromatography. When the chromatographs were developed by spraying with starch-iodide solution, blue spots appeared indicating the presence of oxidizing species (presumably

N-chloro compounds) of good stability in the treated solutions and evaporated residues. When the molar chlorine to cytosine ratio was less than one, only a single spot appeared, at the same distance from the origin for all samples. When the ratio was greater than one, multiple spots appeared, at different distances and having different intensities depending on the initial ratio. The formation of a number of N-chloro derivatives or decomposition products of cytosine is clearly indicated.

Generally speaking the demands exhibited by the amino acids and related compounds can be accounted for semi-quantitatively as an oxidative hydrolysis of N-chlorinated amine groups, followed by breakpoint oxidation of the released ammoniacal-N to nitrogen gas. The greater part of the demand is therefore attributable to oxidation of nitrogen rather than oxidation or substitution on carbon by the aqueous chlorine. Only when the demand exceeds 2.5 moles of chlorine per mole of compound is it likely that any general oxidative breakdown of the organic compound has occurred.

Three of the five compounds exhibiting molar demands much in excess of 2.5, pyrrole, indole and tryptophane, are structurally related in that all possess the pyrrole ring. It appears that the presence of this ring, which is a component of such important natural products as porphyrin, chlorophyll and hemoglobin, may provide a point of attack for general oxidation by aqueous chlorine. Even so, the demands shown do not represent complete oxidation to CO_2 and N_2.

The strong demand exhibited by m-aminophenol, is noteworthy because of its structural resemblance to resorcinol, m-dihydroxybenzene, which is known to produce substantial chloroform when allowed to react with aqueous chlorine.

Initial Chlorine Demand

Initial chlorine demand ratios (ICDR) defined as the chlorine demand ratio determined for reaction times of approximately 30 seconds are shown in Table 39. It is easily seen that many organic compounds exert substantial chlorine demand within a half a minute after mixing.

Those compounds which exhibited high initial chlorine demands are typically members of one of the following groups: 1. benzenoids with at least one attached hydroxyl group (m-aminophenol); or 2. heterocyclic nitrogenous compounds having a pyrrole or pyrrolidine structure (tryptophane, indole and proline).

Long Term Chlorine Demand

Several compounds were chosen for aqueous chlorination studies that lasted a minimum of 20 hours. Table 40 lists these compounds together with highest chlorine demand observed after many hours of reaction with HOCl.

Compounds that exhibited a high initial chlorine demand, such as tryptophane, frequently underwent subsequent slow increase in chlorine demand over time. In the majority of experiments on compounds of this type, the greater part of the total chlorine demand was exerted within the first 30 seconds or so of reaction.

TABLE 39. COMPOUNDS SCREENED FOR INITIAL CHLORINE DEMAND

Compound	Initial chlorine concentration (mM)	HOCl/Cpd[a]	ICDR[b]
alanine	0.20	2:1	ns[c]
m-aminophenol	0.60	6:1	4.50
arabinose	0.05	5:1	0.46
arginine	0.20	2:1	ns
aspartic acid	0.10	3:1	ns
glutamic acid	0.10	1:1	ns
indole	0.20	4:1	1.04
proline	0.30	3:1	2.93
tryptophane	0.40	4:1	2.90

[a] Cpd = the added initial molar concentration of compound in the reaction mixture

[b] ICDR = initial chlorine demand ratio

[c] ns = no significant chlorine demand (<0.25 ICDR)

TABLE 40. HIGHEST CHLOROFORM RATIOS OF SELECTED COMPOUNDS

Compound	Initial Chlorine Concentration (mM)	HOCl/Cpd	Time (hours)	CDR
alanine	0.20	4:1	72	1.73
m-aminophenol	0.80	8:1	41	7.18
arginine	0.20	4:1	72	2.56
aspartic acid	0.20	4:1	72	3.50
caffeine	0.10	2:1	67	0.62
creatinine	0.05	5:1	29	3.28
dimethylaminobenzaldehyde	0.04	4:1	33	3.80
glutamic acid	0.20	4:1	72	2.05
indole	0.40	8:1	31	7.40
phenylalanine	0.20	4:1	67	3.87
proline	0.20	4:1	72	2.76
pyrrole	0.20	13.9:1	24	12.67
tryptophane	0.40	8:1	72	8.00

Chloroform Formation

The stoichiometry of chlorine demand as it relates to chloroform formation shows that each molecule of chloroform produced reduces the theoretical chlorine demand for oxidation to CO_2 and water by one molecule of HOCl. Equations can be written for each chloroform-producing substance to give a maximum expected stoichiometric or theoretical chlorine demand (TCD, noted by underlined values). These are shown in Table 41. Actual demand figures can approach these stoichiometric ones only if the rest of the organic compound is oxidized fully to CO_2 and H_2). (The values for the N-containing compounds are somewhat uncertain. Breakpoint oxidation to N_2 has been assumed, but if oxidation proceeds all the way to NO_3^-, then the theoretical figure should be greater by 2.5 per N atom.) When this happens, there is no organic matter left in the form of additional chloroorganic compounds, except possibly trichloroacetate or hexachloroacetone. In these circumstances the supposition that substantial quantities of many chloroorganic substances besides chloroform have been produced is simply not tenable.

The percent theoretical chlorine demand utilized (%TCD) can be evaluated using equation (15) from the observed chlorine demand ratio and the theoretical chlorine demand ratio.

$$\underset{\text{(\% TCD)}}{\text{\% Theoretical chlorine demand}} = \frac{\text{observed chlorine demand ratio (CDR)}}{\text{theoretical chlorine demand ratio (TCDR)}} \times 100 \qquad (15)$$

Chlorine demand ratios (CDR), % theoretical chlorine demand values (%TCD) along with the highest concentration of chloroform produced during initial studies at pH 7.0 are shown in Table 42. The percent molar yield of $CHCl_3$ is defined by:

$$\text{\% Molar Yield of } CHCl_3 = \frac{\text{moles of chloroform formed}}{\text{moles of compound used}} \times 100 \quad . \qquad (16)$$

It is noteworthy that in all the investigations of chloroform formation the percent molar yields were not greater than 100%, even with the most productive compounds under the most favorable conditions.

The relationship between the percent molar yield of chloroform formed during the 16:1 sample run using tryptophane, and the associated chlorine-demand ratio over a contact period of about 8 hours is depicted in Figure 122. The percent molar yields and chlorine demand ratios for indole, a degradation product of tryptophane, exhibit a similar relationship, but with markedly reduced molar yield of chloroform as compared with that of tryptophane. Figure 123 shows the results of chlorination studies at pH 7 on indole and tryptophane with initial aqueous chlorine concentrations of 1.0×10^{-5}M and molar ratio or HOCl to compound equal to 10.

Several nitrogenous organic compounds, including some with pyrrolic rings, are effective producers of chloroform. Detailed results with 5×10^{-6}M tryptophane, generally typical of findings with the other nitrogenous compounds

TABLE 41. STOICHIOMETRIC EQUATIONS OF COMPOUNDS HAVING CHLOROFORM FORMING POTENTIAL[a]

Compound	+ HOCl	→ $CHCl_3$	+ CO_2	+ H_2O	+ HCl	+ N_2
adenine: $C_5H_5N_5$	11.5	1	4	3.5	8.5	2.5
Alanine: $C_3H_7NO_2$	6.5	1	2	4.5	3.5	0.5
4-amino antipyrine: $C_{11}H_{13}N_3O$	26.5	1	10	7.5	23.5	1.5
m-aminophenol: C_6H_7NO	13.5	1	5	4.5	10.5	0.5
arginine: $C_6H_{14}N_4O_2$	15	1	5	7	12	2
aspartic acid: $C_4H_7NO_4$	6.5	1	3	4.5	3.5	0.5
barbituric acid: $C_4H_4N_2O_3$	6	1	3	3	3	1.0
caffeine: $C_8H_{10}N_4O_2$	18	1	7	6	15	2.0
creatine: $C_4H_7N_3O$	9.5	1	3	4.5	6.5	1.5
cyanuric acid: $C_3H_3N_3O_3$	3.5	1	2	2.5	0.5	1.5
cytosine: $C_4H_5N_3O$	8.5	1	3	3.5	5.5	1.5
p-dimethylaminobenzaldehye: $C_9H_{11}NO$	21.5	1	8	6.5	18.5	2.5
glutamic acid: $C_5H_9NO_4$	9.5	1	4	5.5	6.5	0.5

(continued)

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[a] Number represents moles required for balancing equations.

TABLE 41 (continued)

Compound	+ HOCl	→ $CHCl_3$	+ CO_2	+ H_2O	+ HCl	+ N_2
glycylaglycine: $C_4H_8N_2O_3$	8	1	3	5	5.0	1.0
histidine: $C_6H_9N_3O_2$	13.5	1	5	5.5	10.5	1.5
L-hydroxyproline: $C_5H_9NO_3$	10.5	1	4	5.5	7.5	0.5
indole: C_6H_7N	18.5	1	7	4.5	15.5	0.5
phenylalanine: $C_9H_{11}NO_2$	20.5	1	8	6.5	17.5	0.5
proline: $C_5H_9NO_2$	11.5	1	4	5.5	8.5	0.5
purine: $C_5H_4N_4$	11	1	4	3	8	2.0
pyrrole: C_4H_5N	9.5	1	3	3.5	6.5	0.5
sarcosine: $C_3H_7NO_2$	6.5	1	2	4.5	3.5	0.5
thymine: $C_5H_6N_2O_2$	10	1	4	4	7	1.0
tryptophane: $C_{11}H_{12}N_2O_2$	25	1	10	7	22	1.0
tyrosine: $C_9H_{11}NO_3$	19.5	1	8	6.5	16.5	0.5
uracil: $C_4H_4N_2O_2$	7	1	3	3	4	1
uric acid $C_5H_4N_4O_3$	8	1	4	3	5	2

TABLE 42. HIGHEST CONCENTRATION OF CHLOROFORM PRODUCED DURING INITIAL CHLOROFORM PRODUCTION STUDIES AT pH 7[a]

Compound	Time (hours)	Chlorine Demand Ratio	Theoretical Demand Ratio (Equation 15)	Molar Yield $CHCl_3$ (%)
adenine	4.0	5.7	50	ns[b]
alanine[c]	25	3.0	46	1.0
m-aminophenol	3.8	4.6	34	ns
barbituric acid	4.4	4.3	72	1.4
caffeine	3.3	1.8	10	ns
chlorophyll	4.0			10 μg/l
creatinine	3.7	1.2	16	0.5
cytosine	5.0	3.6	42	0.5
L-hydroxyproline	98	8.5	81	6.9
(pH 10.5)	98	8.5	81	37.2
indole[d]	25	8.5	46	4.9
phenylalanine[c]	24	2.5	12	1.1
proline	3.7	1.6	14	0.6
purine	2.8	.7	6	ns
pyrrole	0.3	3.1	33	0.7
thymine	3.4	5.4	54	0.5
tryptophane[d]	24	10.0	40	7.8
(pH 7.5)[e]	7.0	13.4	54	17.9
tyrosine	4	9.0	46	ns
uracil	0.7	4.3	61	0.6
uric acid	2.0	6.1	76	ns

[a] Initial chlorine concentration of 9.0×10^{-5}M and HOCl/Cpd ratio of 9:1 except where noted.

[b] ns = less than 0.5% molar yield of $CHCl_3$

[c] Initial chlorine concentration 20×10^{-5}M; HOCl/Cpd = 10:1

[d] Initial chlorine concentration 10×10^{-5}M; HOCl/Cpd = 10:1

[e] Initial chlorine concentration 12×10^{-5}M; HOCl/Cpd = 12:1

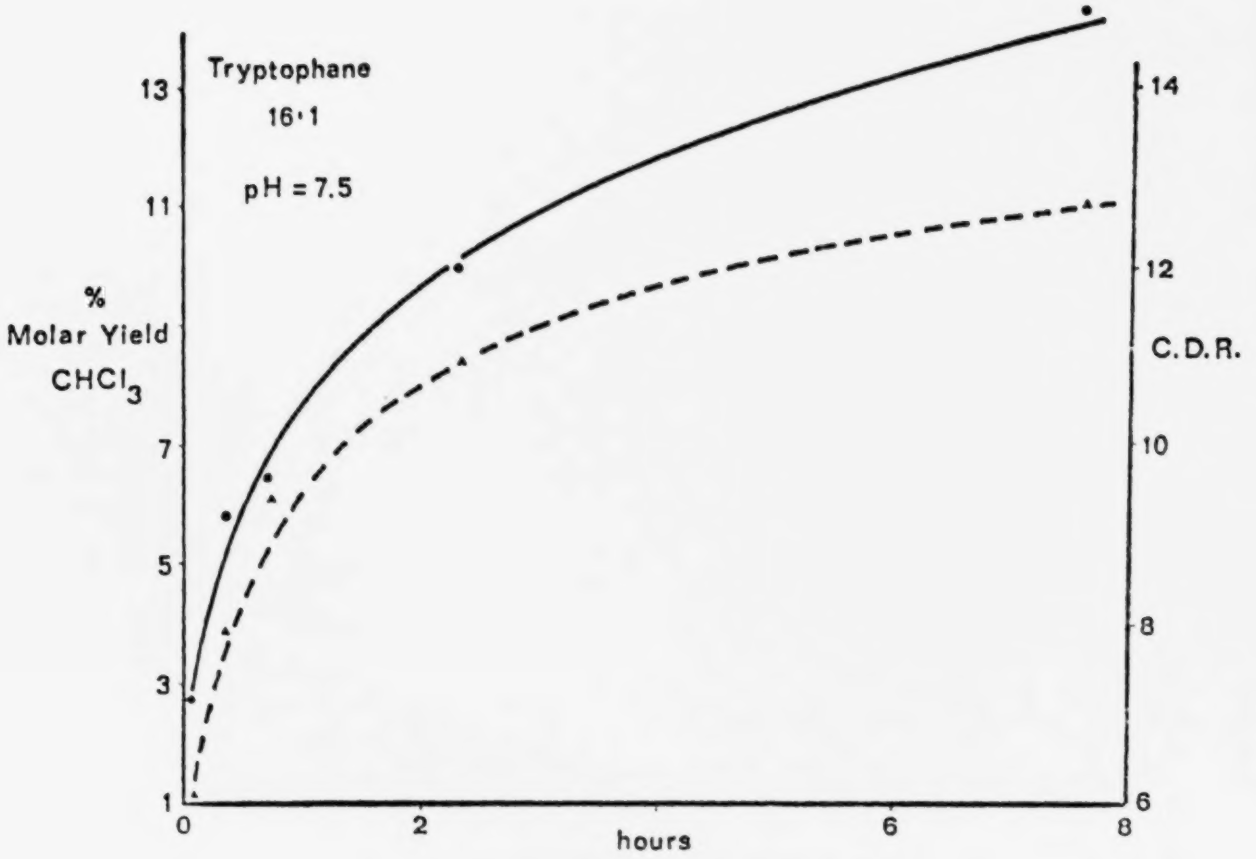


Figure 122. Chlorination of tryptophane, pH. 7.5. Solid line, chloroform formed; dashed line, molar chlorine demand ratio. Initial tryptophane, 0.50×10^{-5}M; initial aqueous chlorine, 8×10^{-5}M (5.7 mg/1).

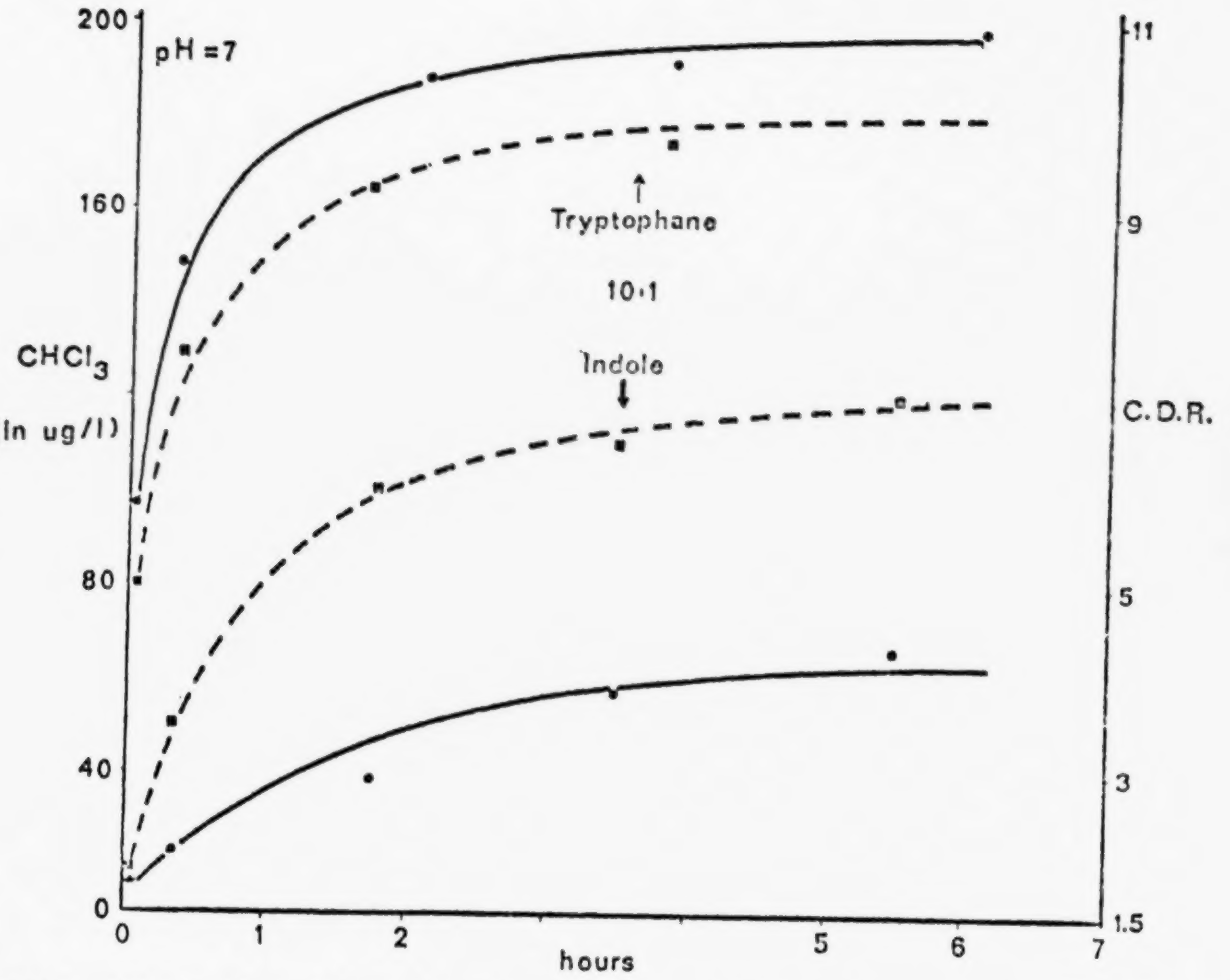


Figure 123. Chlorination of tryptophane and indole, pH 7. Solid lines, chloroform formed; dashed lines, molar chlorine demand ratio. Initial tryptophane, 1.0×10^{-5}M; initial aqueous chlorine, 1.0×10^{-4}M (7.1 mg/l). Initial indole, 1.0×10^{-5}M; initial aqueous chlorine, 1.0×10^{-4}M (7.1 mg/l).

are depicted in Figure 124. An increased chloroform production resulting from increases in the ratio of the chlorine to tryptophane applied was observed. Pyrrole and L-hydroxyproline exhibited similar behavior.

Perhaps most interesting of all were the results with chlorophyll. "Soluble" chlorophyll at a nominal concentration of 1.7 mg per l (the material was an aqueous paste with solids contents unknown) was mixed with 5.7×10^{-4}M (40 mg per l) of aqueous chlorine at pH 5.8, 6.6, 7.0, 9.2 and 10.0 and allowed to stand for about 100 hours. The chloroform produced, in μg per l, was found to be 12, 32, 56, 260 and 230, respectively. A plot of the chloroform produced as a function of pH is shown in Figure 125.

The fact that chlorophyll, even if only at elevated pH, was able to produce such substantial quantities of chloroform is strongly suggestive that algae as well as fulvates may be sources of haloforms in the treatment of water supplies.

Effects of pH Alteration

One of the most significant findings of these studies was the increased yields of chloroform obtained when reaction mixtures were made alkaline an hour or two before analyses for chloroform were performed. Since these increases were observed without significant changes in chlorine consumption and in some instances even when solutions had been dechlorinated prior to pH change, they are strongly indicative of the presence of intermediate chlorinated compounds like trichloroacetate that require alkaline conditions for hydrolysis.

Examples of the results obtained are shown in Figures 126 through 130 for a number of different nitrogenous compounds.

Results for proline are shown in Figure 126 along with values of the molar chlorine-demand ratio at the time of the chloroform determinations. Although increasing the pH produced some increased chlorine demand, the final value was the same for the region pH 9 to 11, the region in which the great increase in yield of chloroform occurred. This was indicative that the greater chloroform production at higher pH was not a result of greater chlorination.

Detailed results for tryptophane are depicted in Figure 127. Perhaps the most remarkable feature in this experiment was the occurrence of 100% molar yield of $CHCl_3$ near pH 11. This is to be compared with a maximum observed yield of 18% in the studies at pH 7.5. It becomes clear, once again, that chlorination to intermediate products occurs during the reaction period in neutral or mildly acidic solutions, with $CHCl_3$ being liberated by hydrolysis when the pH is raised.

Chlorination reactions with parallel samples of tryptophane and indole conducted over a period of 28 hours at pH 6 yield considerably different molar percentages of chloroform, about 14% for tryptophane and 3% for indole. This is shown by Figure 128. After the 28 hours of contact, the pH of each sample was sequentially increased by addition of increments of sodium

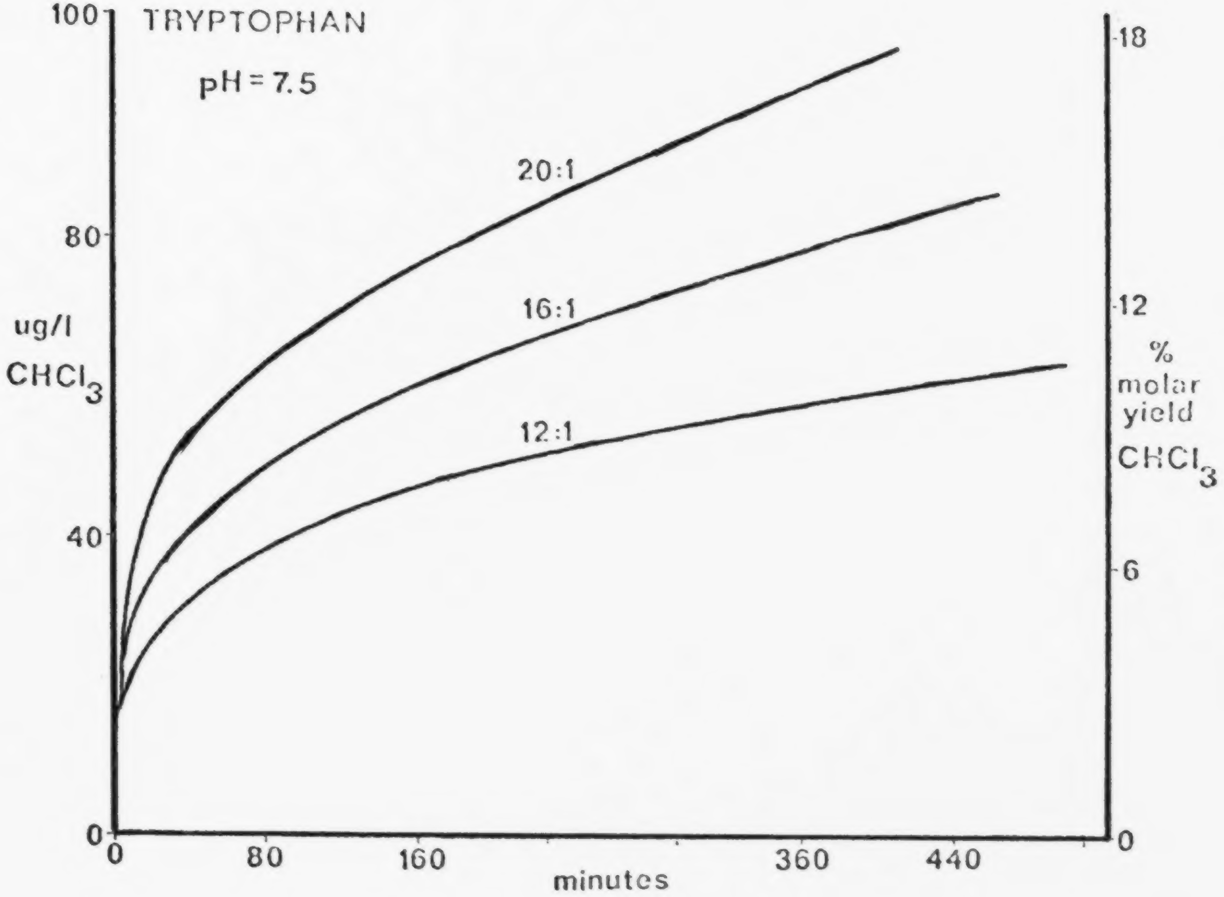


Figure 124. Chlorination of tryptophane, pH 7.5. Initial tryptophane concentration, 0.50×10^{-5}M; initial chlorine concentrations, 6.0×10^{-5}M (4.3 mg per l), 8×10^{-5}M (5.7 mg per l), 1.0×10^{-4}M (7.1 mg per l).

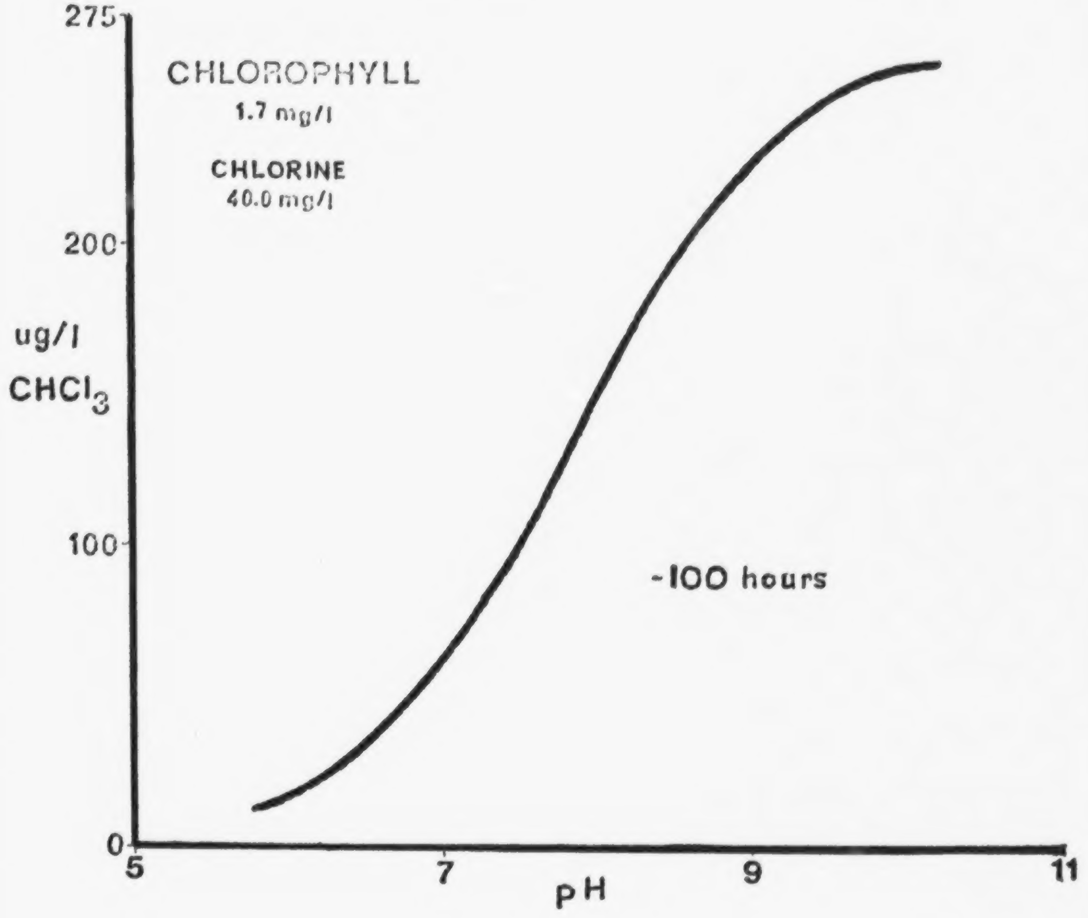


Figure 125. Chlorination of chlorophyll at varied pH. Initial chlorophyll concentration (nominal), 1.7 mg per 1; initial chlorine, 5.7×10^{-4}M (40 mg per 1). pH values, 5.8, 6.6, 7.0, 9.2, 10.0. Chlorine demand after 100 hours 36 mg per 1 in all solutions.

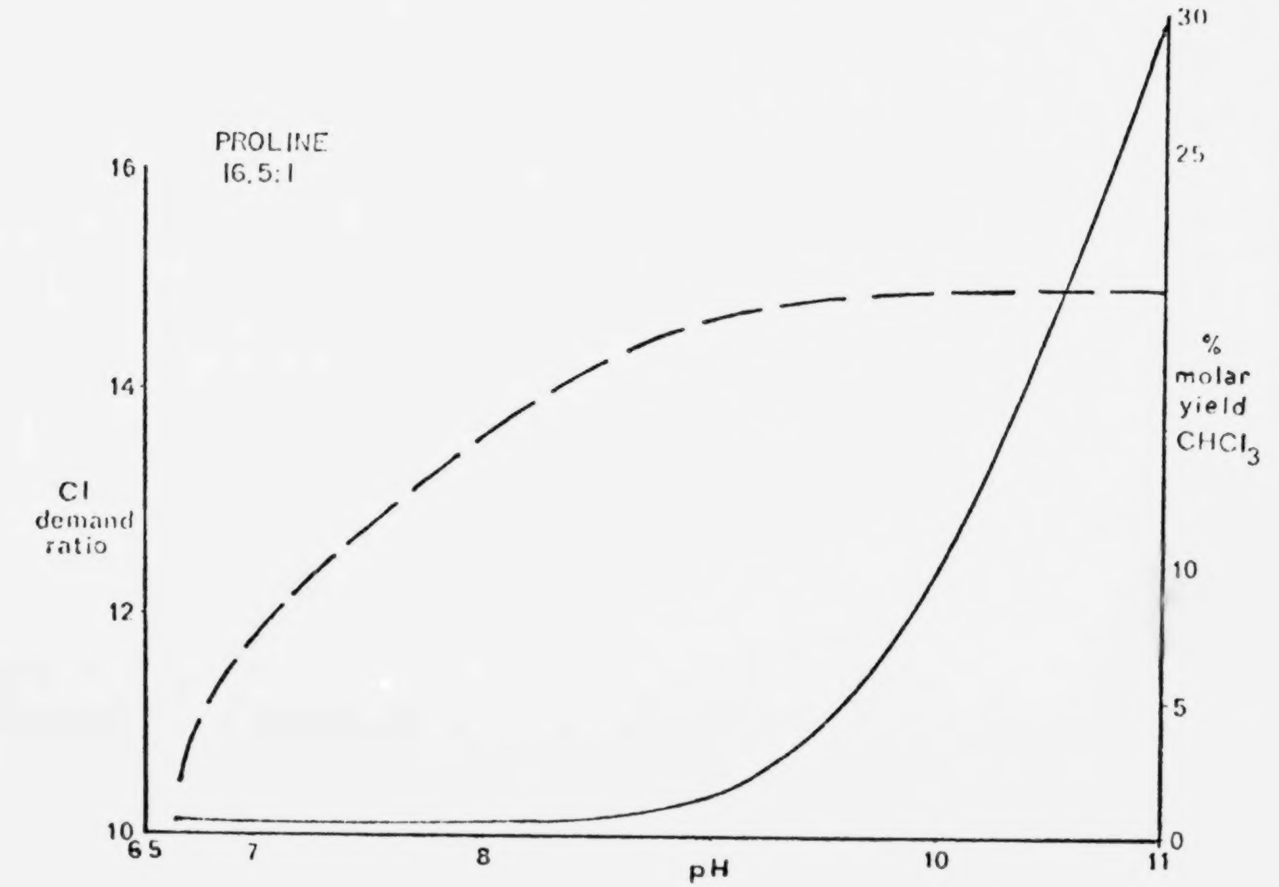


Figure 126. Yields of $CHCl_3$ from proline with pH change. Solid line, chloroform yield; dashed line, molar chlorine-demand ratio. Initial proline concentration, 2.0×10^{-5}M; initial aqueous chlorine, 3.3×10^{-4}M (23 mg per 1). pH at 6.7 for 21 hours, then separate samples increased in about 0.5 pH unit steps with greatest pH at 11. Chloroform and residual chlorine determinations at 24 hours.

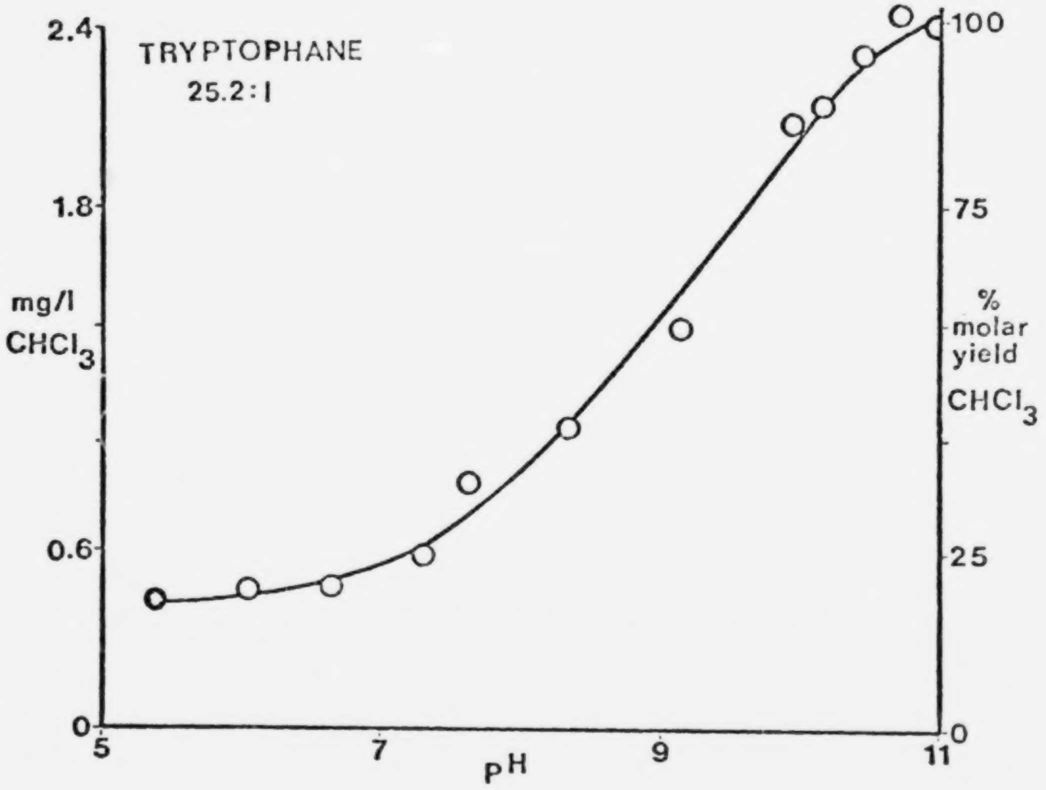


Figure 127. Yields of chloroform from tryptophane with pH change. Initial tryptophane concentration, 2.0×10^{-5}M; initial aqueous chlorine, 5.04×10^{-4}M (36 mg/l). pH at 5.4 for 45 hours, then separate samples increased in pH in 0.5 unit step with greatest pH near 11. Chloroform determinations at 47 hours.

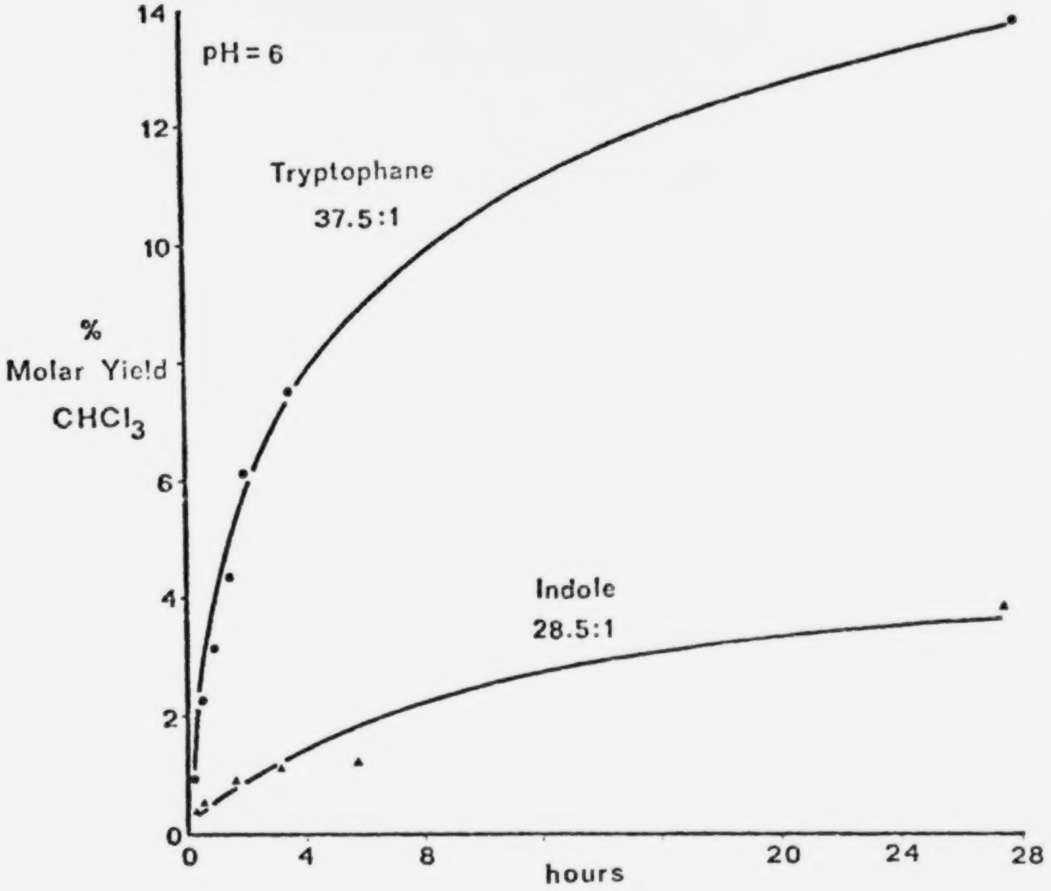


Figure 128. Chlorination of tryptophane and indole, pH 6. Initial tryptophane, 1.0×10^{-5}M; initial aqueous chlorine, 3.75×10^{-4}M (26.5 mg/l). Initial indole, 1.0×10^{-5}M; initial aqueous chlorine, 2.85×10^{-4}M (20.2 mg/l).

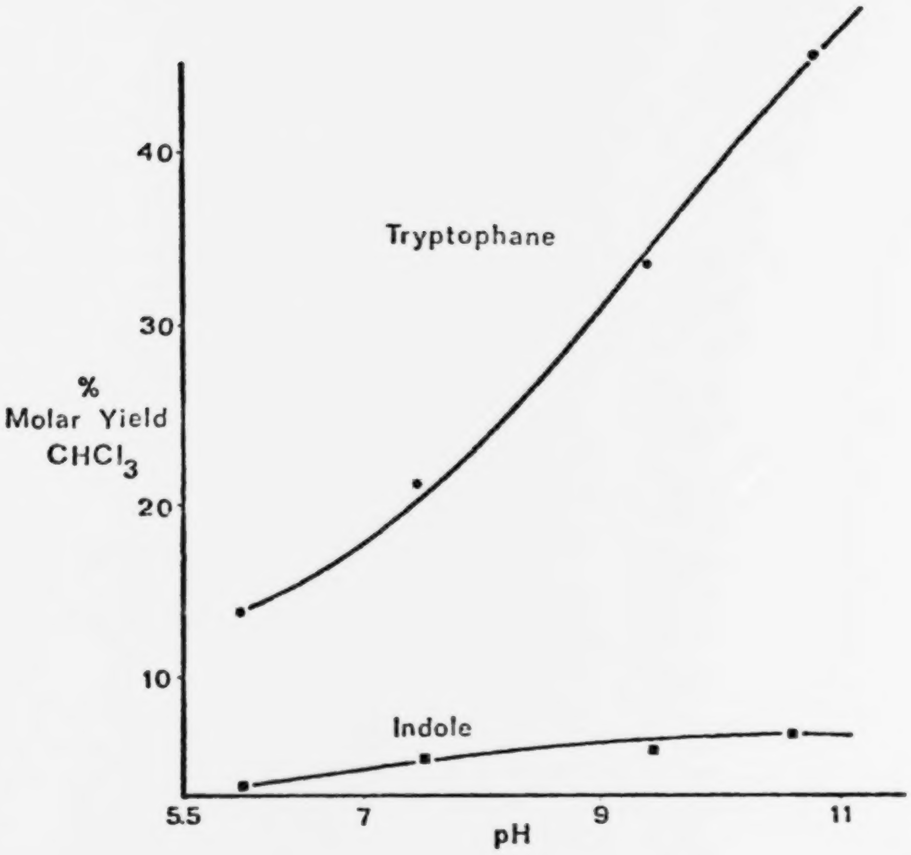


Figure 129. Yields of chloroform from tryptophane and indole with pH change. Yields of chloroform from tryptophane and indole with pH change after reduction of residual chlorine with thiosulfate after 28 hours at pH 6. Initial tryptophane, 1×10^{-5}M; initial aqueous chlorine 3.75×10^{-4}M (26.5 mg/l). Initial indole, 1×10^{-5}M; initial aqueous chlorine, 2.85×10^{-4}M (20.2 mg/l).

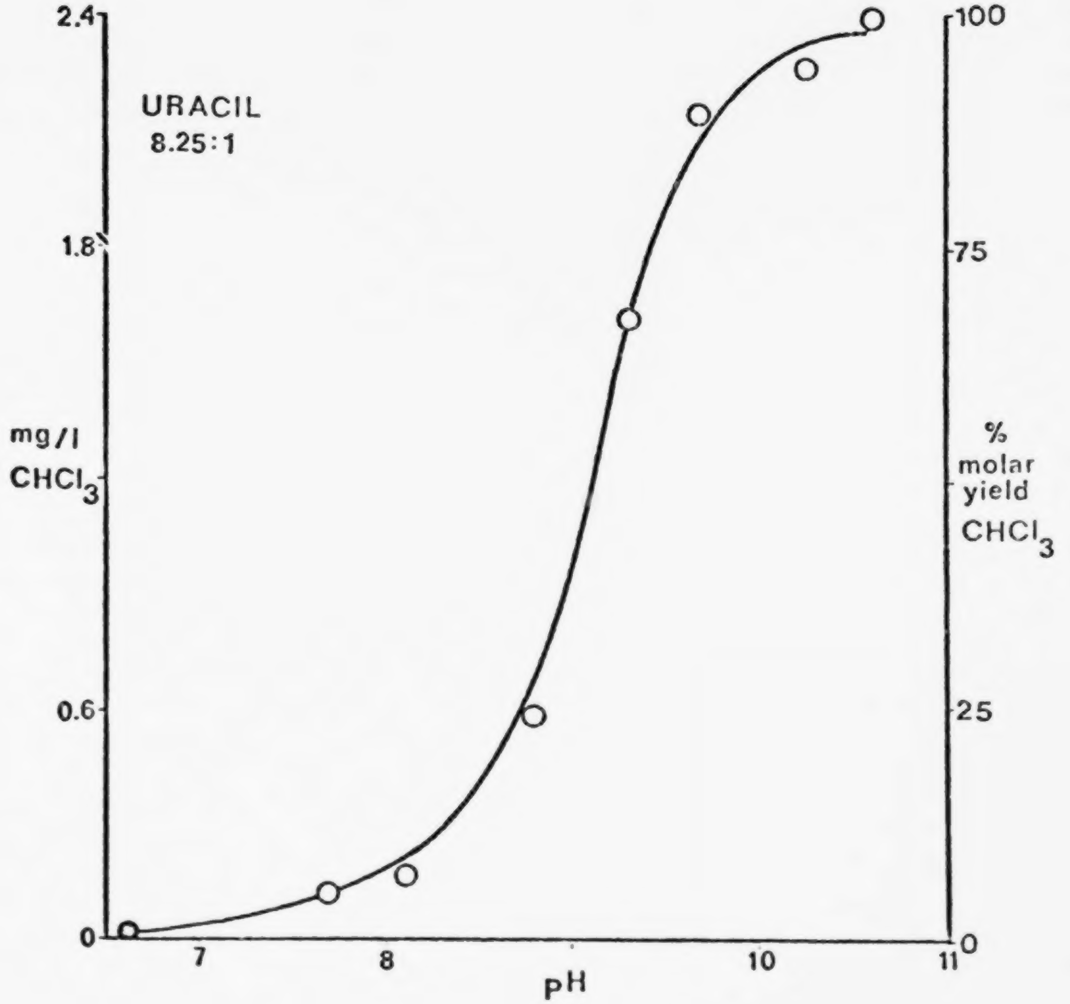


Figure 130. Yields of chloroform from uracil with pH change. Initial uracil concentration, 2.0×10^{-5}M; initial aqueous chlorine, 1.65×10^{-4}M (11.7 mg/l). After reaction at pH 6.6 for 21 hours virtually all chlorine reduced. Separate samples increased in pH to a maximum of 10.6. Chloroform determinations at 26 hours.

hydroxide after removal of any remaining available chlorine by addition of sodium thiosulfate. Within minutes of each pH alteration the concentrations of chloroform were determined. The results, shown in Figure 129, indicate the amount of chloroform obtained from indole does not significantly increase when pH is increased after dechlorination whereas that obtained from tryptophane increases greatly. Since there was no available chlorine remaining to provide additional chlorination, it appears that an intermediate was formed at pH 6 which hydrolyzed or decomposed to give chloroform when sufficient base was added. This observation is consistent with the information derived from the spectrophotometric studies.

A similar pattern was exhibited for the chlorination of uracil with pH change presented in Figure 130. Uracil had not previously been regarded as a significant chloroform producer based on studies near pH 7. This experiment showed conclusively that extensive chlorination had occurred during the reaction, so that when the pH was increased, with virtually no residual available chlorine present. 100% molar yield of chloroform was obtained.

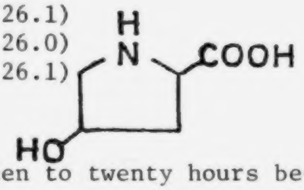
The study of L-hydroxyproline illustrates the differences resulting from different pH values. Parallel samples of L-hydroxyproline were prepared in different buffer systems, one at neutral pH and the other at pH 10.5. Each was then mixed with an equal volume of chlorine solution diluted with the same buffer as the L-hydroxyproline sample. Shoftly after mixing, both solutions exhibited similar chlorine demand and chlorine production. However, after two hours had past, the alkaline sample showed a significantly greater chlorine demand in addition to a more than forty-fold increase in chloroform formation as compared to that of the solution at neutral pH (see Figure 131).

Results of chloroform production by L-hydroxyproline at various pH values are shown below:

L-hydroxyproline	2.0×10^{-5}M	(2.6 ppm)
chlorine	55.5×10^{-5}M	(39.3 ppm)

Approximate time: 170 hours

pH	Molar Yield of Chloroform	Observed Demand Ratio
5.6 (7.0)[a]	0.1% (1.1%)	25.4 (25.9)
(8.2)	(2.4)	(26.1)
(10.0)	(110.0)	(26.1)
6.6 (10.0)	0.6 (96.8)	25.9 (26.0)
7.0 (10.0)	1.5 (89.4)	26.1 (26.1)
9.2	37.2	26.0
10.0	41.5	25.9



[a] Sodium hydroxide was added to the low pH samples fifteen to twenty hours before chloroform determination was carried out at Lawrence. In addition, the sample originally at the lowest pH was separated into four portions: the first was placed in a screw-capped vial without any alteration while each of the other three had sodium hydroxide added until pH values of 7.0, 8.2 and 10.0 were attained at which time they were placed in screw-capped vials for transport to Lawrence.

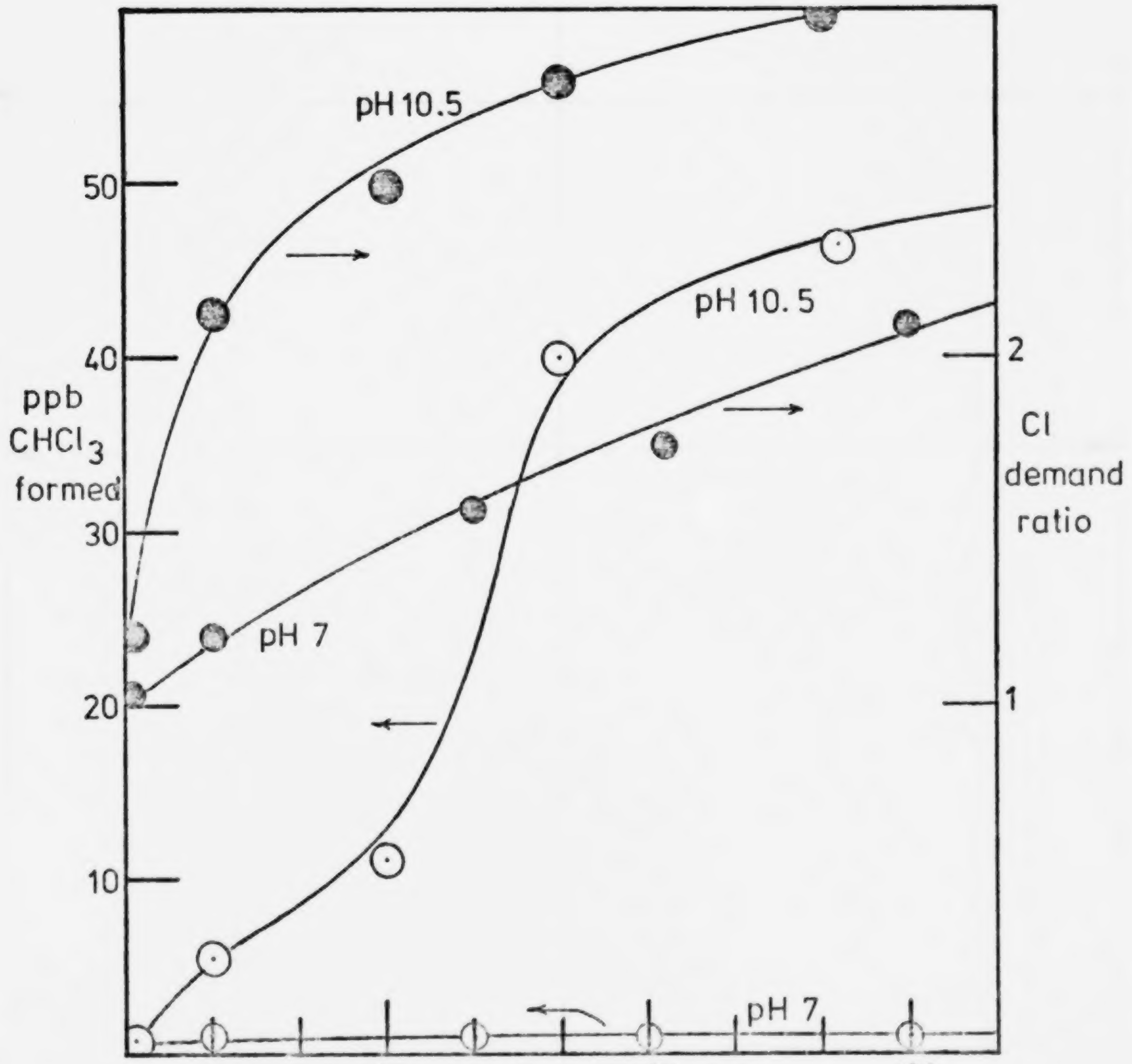


Figure 131. Production of chloroform (o) and chlorine demand ratio (•) for chlorination of 10 x 10^{-5}M L-hydroxyproline with 9 x 10^{-5} M aqueous chlorine at pH 7 and 10.5 (chlorine demand ratio = moles of chlorine used/mole of L-hydroxyproline added).

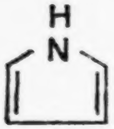
The results of this study were very interesting. As was the case in other compounds, the main increase in chloroform production occurred between pH 7.0 and pH 9.2. However, the subsequent increase, that occurred between pH 9.2 and pH 10.0, was not substantial. This suggested that the chloroform production tended to level off at the higher pH values, or perhaps, as in the case of chlorophyll, the maximum chloroform yield was reached prior to the highest pH. Again, the observed demand ratios were not significantly different for any of the original pH values.

The samples which were increased in pH also produced rather interesting results. The first sample, pH 5.6 exhibited little increase in chloroform formation when raised to pH 8.2. Yet, when the pH reached 10.0 the molar yield of the chloroform exceeded 100%. Apparently, a very dramatic increase occurs at some point between these two pH values. Also, the two other samples which underwent pH adjustment showed significantly greater quantities of chloroform formed than the sample originally at pH 10.0. The changes in demand ratios were negligible. Additional nitrogenous compounds which produced considerable quantities of chloroform under alkaline conditions were pyrrole and m-aminophenol.

Pyrrole: pyrrole 2.0×10^{-5} M (1.3 ppm)
chlorine 25.0×10^{-5} M (17.7 ppm)

Approximate time: 24 hours

pH	Molar Yield of Chloroform	Observed Demand Ratio
5.7	0.2 %	12.0
6.5	0.4	11.9
7.5	0.5	11.9
9.1	7.6	12.0
10.0	30.0	12.0

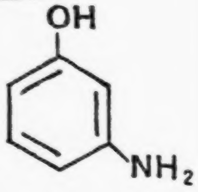


A significant increase in chloroform production occurred between pH 7.5 and pH 9.1 and again between pH 9.1 and pH 10.0. However, it is quite interesting to note that the demand ratios for all pH values were almost identical. This indicated the formation of an intermediate compound which, at the lower pH values, did not readily convert to chloroform

m-Aminophenol: m-aminophenol 2.0×10^{-5} M (2.2 ppm)
chlorine 37.0×10^{-5} M (26.2 ppm)

Approximate time: 150 hours

pH	Molar Yield of Chloroform	Observed Demand Ratio
4.4 (10.1)	3.4 % (17.8%)	17.6 (17.6)
6.3 (10.1)	7.6 (12.5%)	17.6 (17.6)
6.8 (10.1)	13.4 (16.2%)	17.6 (17.6)
9.2	17.3	17.7
9.9	30.8	17.7



In the case of m-aminophenol, the first large increase in chloroform formation occurred between pH 6.3 and pH 6.8, the next occurring between pH 9.2 and pH 9.9 (Figure 132). As observed previously, the difference among the demand ratios was not significant. In addition, even after the acidic samples were raised to pH 10.1 their demand ratios (determined by thiosulfate titration after gas chromatography analysis) did not change. On the other hand, the amount of chloroform produced increased markedly. This substantiated the previous hypothesis that an intermediate was formed initially, but its conversion to chloroform was inhibited due to the low pH of some of the samples. When the pH values were subsequently increased, the formation of chloroform was favored. Also, the lowest pH sample produced the greatest amount of chloroform after the pH was raised. This might mean that formation of the intermediate (or intermediates) was favored at the lowest pH value.

A possible explanation for the striking chloroform production of the nitrogenous compounds discussed is that heterocyclic structures often react in a manner similar to ketones (100). The elevated pH results in rapid tautomerism to the enol form, thus allowing the reaction to proceed analogously to that of diketo compounds. These diketo compounds have been known to produce significant amount of chloroform when allowed to react with aqueous chlorine (35).

Although altering the pH and concentrations can result in different values of chlorine demand and quantities of chloroform formed, there are other variables which can also affect these values. Recent studies conducted at the Lawrence Experiment Station on chloroform concentrations in Massachusetts drinking water supplies indicate that contact time is one such important parameter. In this investigation all sample runs were carried out over a period of several hours with demand and concentration of chloroform being determined periodically for extrapolation necessitated by time constraints on the use of the equipment. Other variables, such as temperature, were not investigated in this study.

CONCLUSIONS

A large number of naturally occurring nitrogenous compounds readily reacted with aqueous chlorine exerting significant chlorine demands. The groups of compounds found to be moderately to highly reactive included: benzoids containing one or more hydroxyl groups (m-aminophenol), amino acids (alanine, tryptophane, proline and L-hydroxyproline); structures containing the pyrrole ring (indole, pyrrole, chlorophyll and some amino acids already mentioned); pyrimidines (cytosine, uracil, thymine and barbituric acid); and some purines (adenine and uric acid). Not all organic compounds that exhibited high chlorine demand produced chloroform as a product suggesting either simple oxidation or the formation of other chlorinated organic compounds which have so far remained unidentified. For those compounds which did produce chloroform the chlorine demand did not appear to be a very good indication of the chloroform producing potential. For chloroform producers, increasing the pH had a considerable influence on the quantity of chloroform formed, with maximum chloroform formation occurring between pH 8.5 and pH 10.5.

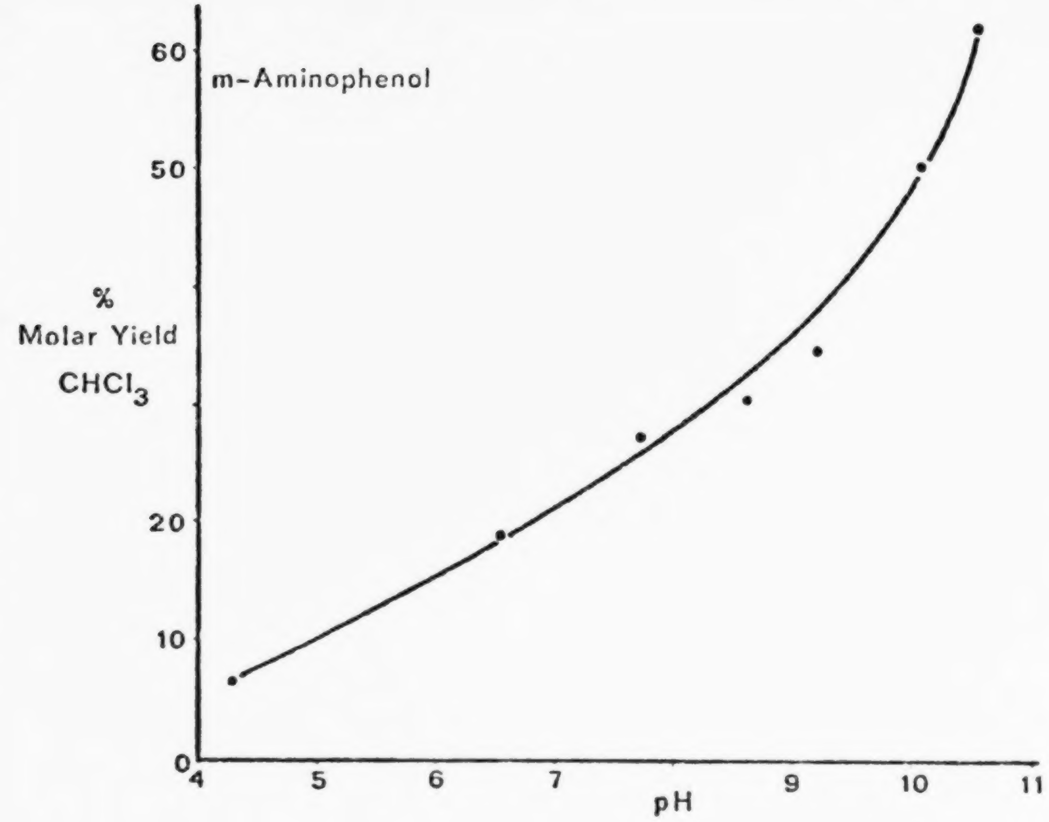


Figure 132. Yields of chloroform from m-aminophenol with pH change. Initial m-aminophenol, 2×10^{-5}M; initial aqueous chlorine, 3.3×10^{-4}M (23 mg/l). pH at 4.3 for 41 hours, then pH increased. Chloroform determinations at 67 hours.

Compounds that reacted with aqueous chlorine near pH 7 to yield chloroform did not necessarily exhibit enhanced production of chloroform with increase in pH. Moreover, several compounds that showed sharp increases in chloroform formation under alkaline conditions also began to show decreases in chloroform production with increasing pH values greater than about 10. Thus, aqueous chlorine reactions of selected compounds (or raw waters) having pH values greater than 10 can give misleading indications of the ultimate capacity for formation of chloroform.

Some compounds that showed significant chloroform production only under alkaline conditions generally formed other chlorinated intermediates under neutral or slightly acidic conditions. These intermediates when subjected to a higher pH, produced chloroform. Samples for haloform should, therefore, be made alkaline to pH 11 or greater and held at the pH for an hour or so before haloform determinations.

It seems possible that a very simple technique for preliminary assessment of the potential for chloroform production in a water supply can be obtained by determination of the four to six-hour chlorine demand with enough excess chlorine present to give a free residual chlorine of several milligrams per liter.

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APPENDIX A

TABLE A-1. RECOVERY OF CREATINE IN WATER AFTER PASSAGE THROUGH TENAX AND XAD-8 MACRORETICULAR RESINS (pH UNADJUSTED)

aliquot (ml)	absorbance at 254 nm[a]	% recovery
influent (= 10 mg/L)	0.600	-
0-25	0.033	5.5
25-30	0.035	5.8
30-35	0.064	10.7
35-40	0.109	18.2
40-45	0.157	26.2
45-50	0.204	34.0
50-55	0.257	42.8
55-60	0.308	51.3
60-65	0.360	60.0
65-70	0.405	67.5
70-75	0.450	75.0
75-80	0.468	78.0
80-85	0.491	81.8
85-90	0.510	85.0
90-95	0.535	89.2
95-100	0.542	90.3
100-105	0.550	91.7
105-110	0.553	92.2
110-116	0.568	94.7
116-121	0.575	95.8
121-126	0.568	94.7
126-146	0.581	96.8
146-166	0.578	96.3
166-186	0.585	97.5
186-206	0.581	98.5
206-226	0.582	97.0
226-246	0.581	98.5
246-266	0.595	99.2

[a] 1 cm pathlength

TABLE A-2. RECOVERY OF URACIL IN WATER AFTER PASSAGE THROUGH TENAX AND XAD-8 MACRORETICULAR RESINS (pH UNADJUSTED)

aliqout (ml)	absorbance at 254 nm[a]	% recovery
influent = 10 mg/L	.715	-
0-25	.009	1.3
25-30	.026	3.6
30-35	.053	7.4
35-40	.147	20.6
40-45	.250	35.0
45-50	.364	50.9
50-55	.472	66.0
55-60	.510	71.3
60-65	.508	71.1
65-70	.629	88.0
70-75	.645	90.2
75-80	.659	92.2
80-85	-	-
85-91	.680	95.1
91-96	.681	95.2
96-101	.681	95.2
101-106	.690	96.5
106-111	.690	96.5
111-116	.692	96.8
116-121	.692	96.8
121-126	.692	96.8
126-152	.705	98.6
152-177	.725	101.4
177-202	.720	101.7
202-227	.715	100.0
302-326	.715	100.0

[a] 1 cm pathlength

TABLE A-3. RECOVERY OF URACIL IN WATER AFTER PASSAGE THROUGH XAD-8 MACRORETICULAR RESIN (pH UNADJUSTED)

aliquot (ml)	absorbance at 254 nm[a]	% recovery
Influent (= 10 mg/L)	.690	-
0-20	.018	2.6
20-25	.064	9.3
25-31	.126	18.3
31-36	.264	38.3
36-41	.330	47.8
41-46	.410	59.4
46-51	.480	69.6
51-56	.541	78.4
56-62	.569	82.5
62-67	.599	86.8
67-72	.612	88.7
72-77	.640	92.8
77-82	.649	94.1
82-87	.649	94.1
87-92	.655	94.9
92-97	.660	95.7
97-102	.662	95.9
102-107	.651	94.4
107-122	.651	94.4
112-117	.640	92.8
117-122	.672	97.4
122-147	.658	95.4
147-172	.678	98.3
297-322	.687	98.3

[a] 1 cm pathlength

TABLE A-4. RECOVERY OF CREATININE IN WATER ADJUSTED TO pH 2.0 WITH HCl AFTER PASSAGE THROUGH XAD-2 AND XAD-4 RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	3.2	-
0-5	1.5	47.7
5-10	1.1	34.6
10-15	1.5	47.1
15-20	1.6	49.0
20-25	1.9	58.2
25-30	2.4	75.8
30-40	2.2	68.6
40-65	2.5	74.5
65-90	2.5	80.0
90-190	2.6	81.0

TABLE A-5. RECOVERY OF CYTOSINE IN WATER AFTER PASSAGE THROUGH XAD-2 XAD-4 MACRORETICULAR RESINS (pH UNADJUSTED)

aliquot (ml)	concentration (mg/L)	% recovery
influent	8.6	-
0-5	0.7	7.9
5-10	0.4	4.6
10-15	0.9	10.6
15-20	2.0	23.6
20-25	5.3	62.2
25-50	5.4	63.6
75-100	7.1	82.6
175-200	7.5	88.0

TABLE A-6. RECOVERY OF CYTOSINE IN WATER ADJUSTED TO pH 2 WITH NITRIC ACID AFTER PASSAGE THROUGH XAD-2 AND XAD-4 RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	8.3	-
0-5	1.1	13.4
5-10	1.4	11.0
10-15	2.6	31.2
15-20	5.3	63.0
20-25	4.9	59.1
25-30	5.4	64.6
30-40	6.5	77.4
40-65	7.2	85.8
65-90	7.3	87.5
165-190	7.6	90.8

TABLE A-7. RECOVERY OF INDOLE IN 10^{-3} NaOH AFTER PASSAGE THROUGH XAD-2 AND XAD-4 RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	8.7	-
0-5	1.8	20.3
5-10	1.3	20.0
10-15	1.1	12.7
15-20	2.3	26.8
20-25	1.3	14.7
25-50	1.6	18.6
50-75	1.5	17.0
175-200	1.7	19.3

TABLE A-8. RECOVERY OF INDOLE IN 10^{-3} M HNO_3 AFTER PASSAGE THROUGH XAD-4 RESIN

aliquot (ml)	concentration (mg/L)	% recovery
influent	6.6	-
0-5	0.4	6.1
5-10	0	0
10-15	0.3	4.4
15-20	0.1	0.9
20-25	0.1	0.9
25-30	0.1	1.7
30-55	0.1	1.3
55-80	0	0
125-150	0.3	4.8
190-215	0.3	4.8

TABLE A-9. RECOVERY OF CYTOSINE IN WATER AFTER PASSAGE THROUGH XAD-8 AND TENAX RESINS (pH UNADJUSTED)

aliquot (ml)	absorbance at 233 nm[a]	% recovery
influent (= 10 mg/L)	.435	-
0-25	.170	39.1
25-30	.090	20.7
30-35	.111	25.5
35-40	.161	37.0
40-45	.195	44.8
45-50	.236	54.3
50-56	.262	60.2
56-61	.290	66.6
61-66	.323	74.3
66-71	.335	77.0
71-76	.353	81.2
76-81	.365	83.9
81-86	.380	87.4
86-91	.387	89.0
91-96	.392	90.0
96-101	.408	93.8
101-106	.412	94.7
106-111	.414	95.2
111-116	.414	95.2
116-121	.418	96.1
121-126	.413	92.6
126-146	.403	92.6
146-166	.412	94.7
166-186	.416	95.6
186-206	.416	95.6
206-226	.418	96.1
267-287	.418	96.1

[a] 1 cm pathlength

TABLE A-10. RECOVERY OF HUMIC ACID SOLUTION (pH 2.0) AFTER PASSAGE THROUGH XAD-8 AND TENAX MACRORETICULAR RESINS

volume eluted (ml)	absorbance[a] at 330 nm	concentration (mg/L)	% recovery
influent	0.322	13.5	-
0-25	0.070	2.9	21.7
50-75	0.046	1.9	14.3
75-100	0.050	2.1	15.6
100-125	0.050	2.1	15.5
125-175	0.053	2.2	16.5
175-275	0.077	3.2	23.9
275-375	0.090	3.8	28.0
375-474	0.190	4.6	33.9
474-545	0.116	4.9	36.0

[a] 1 cm pathlength

TABLE A-11. RECOVERY OF HUMIC ACID SOLUTION ADJUSTED TO pH 11.7 AFTER PASSAGE THROUGH XAD-8 AND TENAX MACRORETICULAR RESINS

volume eluted (ml)	absorbance at 330 nm[a]	concentration (mg/L)	% recovery
influent	0.438	18.3	-
0-25	-	-	-
25-30	.102	4.3	23.3
30-35	.104	4.4	23.7
35-40	.164	6.9	47.4
40-45	.185	7.8	42.4
45-50	.191	8.0	43.6
50-55	.204	8.5	46.6
55-60	.228	9.6	52.1
60-65	.246	10.3	56.2
65-70	.265	11.1	60.5
70-75	.278	11.6	63.5
75-80	.288	12.1	65.8
80-85	.295	12.4	67.4
85-90	.307	12.9	70.1
90-95	.318	13.3	72.6
95-100	.323	13.5	73.7
100-110	.331	13.9	75.6
110-120	.350	14.7	79.8
120-145	.363	15.2	82.9
145-170	.374	15.7	85.4
170-195	.368	15.4	84.0
195-370	.385	16.1	87.9

[a] 1 cm pathlength

TABLE A-12. RECOVERY OF TRYPTOPHAN IN WATER ADJUSTED TO pH 2.0 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	9.7	-
0-5	0.2	2.4
5-10	0.3	2.7
10-15	0.5	5.4
15-20	0.7	7.1
20-25	1.1	10.9
25-30	1.5	16.0
30-35	2.1	21.8
35-40	2.7	27.9
40-45	3.2	32.7
45-50	3.6	37.4
50-55	3.9	40.5
55-60	4.2	43.9
60-65	4.6	48.0
65-70	4.8	49.7
70-75	5.1	53.1
75-80	5.3	54.4
80-85	5.5	57.1
85-90	5.9	60.5
90-95	6.0	61.6
95-100	6.1	63.6
100-125	6.5	67.7
125-150	7.6	78.9
150-175	7.9	81.6
175-200	8.2	84.8
200-250	8.5	87.8
250-300	8.7	89.8
300-350	8.8	91.5
350-400	8.9	92.5

TABLE A-13. RECOVERY OF TRYPTOPHAN IN WATER ADJUSTED TO pH 6.9 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	absorbance at 278 nm (A.U.)[a]	% recovery
influent	0.365[b]	-
0-5	0.041	11.2
5-10	0.130	35.6
10-15	0.190	52.1
15-20	0.218	59.7
20-25	0.241	66.0
25-30	0.275	75.3
30-35	0.290	79.5
35-40	0.310	84.9
40-45	0.320	87.7
45-50	0.330	90.4
50-55	0.335	91.8
55-60	0.340	93.2
60-65	0.345	94.5
65-70	0.348	95.3
70-75	0.350	95.9
75-80	0.354	97.0
80-85	0.353	96.7
85-90	0.353	96.7
90-95	0.358	98.1
95-100	0.360	98.6
100-125	0.352	96.4
125-150	0.360	98.6
150-175	0.361	98.9
175-200	0.361	98.9
200-250	0.359	98.4
250-300	0.362	99.2
300-350	0.363	99.5
350-400	0.365	100

[a] 1 cm pathlength; H_2O reference

[b] influent concentration = 10 mg/L

TABLE A-14. RECOVERY OF ADENINE IN WATER ADJUSTED TO pH 2.5 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	9.54	-
0-5	0.5	5.0
5-10	2.1	21.8
10-15	3.6	37.6
15-20	4.4	45.8
20-25	5.1	53.1
25-30	5.7	59.3
30-35	6.2	64.6
35-40	6.8	70.9
40-45	7.2	75.0
45-50	7.5	78.1
50-55	7.8	81.2
55-60	8.1	84.4
60-65	8.2	85.4
65-70	8.3	86.5
70-75	8.4	88.5
75-80	8.5	89.5
80-85	8.7	91.1
85-90	8.7	91.6
90-95	8.9	93.7
95-100	8.9	93.7
100-125	9.0	94.8
125-150	9.2	96.9
150-175	9.4	99.0
175-200	9.3	97.9
200-250	9.3	97.9
250-300	9.4	99.0
300-350	9.4	99.0

TABLE A-15. RECOVERY OF ADENINE IN WATER ADJUSTED TO pH 7.1 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	9.6	-
0-5	0.0	0.3
5-10	1.0	10.6
10-15	2.5	25.7
15-20	3.7	37.9
20-25	4.4	46.1
25-30	5.1	53.1
30-35	5.9	60.8
35-40	6.4	66.0
40-45	6.9	71.7
45-50	7.4	76.3
50-55	7.5	77.3
55-60	7.6	78.4
60-65	7.8	80.4
65-70	8.1	83.5
70-75	8.3	86.6
75-80	8.5	88.7
80-85	8.7	90.2
85-90	8.8	91.8
90-95	8.9	92.8
95-100	8.9	92.8
100-125	8.9	72.8
125-150	9.3	96.9
150-175	9.4	97.9
175-200	9.4	97.9
200-250	9.3	96.9
250-300	9.5	99.0
300-350	9.6	100
350-400	9.6	100

TABLE A-16. RECOVERY OF 5-CHLOROURACIL IN WATER ADJUSTED TO pH 2.0 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH CAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	9.7	-
0-5	0.2	2.3
5-10	1.2	16.0
10-15	3.1	31.9
15-20	3.5	36.5
20-25	3.9	40.0
25-30	4.4	45.2
30-35	5.0	51.7
35-40	5.6	58.1
40-45	6.3	65.0
45-50	6.8	70.4
50-55	7.3	75.4
55-60	7.7	79.2
60-65	8.0	82.3
65-70	8.2	84.2
70-75	8.4	86.5
75-80	8.6	88.7
80-85	8.8	90.2
85-90	8.9	91.5
90-95	8.9	92.3
95-100	9.0	93.1
100-125	9.2	94.4
125-150	9.5	97.7
150-175	9.5	98.5
175-200	9.7	100
200-250	9.7	99.6
250-300	9.7	100
300-350	9.7	100
350-400	9.7	100

TABLE A-17. RECOVERY OF 5-CHLOROURACIL IN WATER ADJUSTED TO pH 7.01 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	10.4	-
0-5	0.5	4.5
5-10	2.0	18.9
10-15	3.5	33.2
15-20	4.7	44.6
20-25	5.5	52.9
25-30	6.3	60.7
30-35	7.1	67.9
35-40	7.7	74.1
40-45	8.2	78.7
45-50	8.6	82.1
50-55	8.9	89.8
55-60	9.1	87.5
60-65	9.3	89.3
65-70	9.5	91.1
70-75	9.6	92.0
75-80	9.6	92.6
80-85	9.7	92.9
95-90	9.9	94.6
90-95	9.9	94.6
95-100	10.0	95.5
100-125	10.0	95.5
125-150	10.3	98.2
150-175	10.3	99.1
175-200	10.4	99.6
200-250	10.2	97.3
250-300	10.3	99.1
300-350	10.3	99.1
350-400	10.4	100

TABLE A-18. RECOVERY OF SUCCINIMIDE IN WATER ADJUSTED TO pH 2.0 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	absorbance at 215 nm (A.U.)[a]	% recovery
influent	1.000[b]	-
0-10	0.127	12.7
10-20	0.196	19.6
20-30	0.245	24.5
30-40	0.318	31.8
40-50	0.383	38.3
50-60	0.435	43.5
60-70	0.470	47.0
70-80	0.540	54.0
80-90	0.550	55.0
90-100	0.590	59.0
100-125	0.620	62.0
125-150	0.690	69.0
150-175	0.780	78.0
175-200	0.810	81.0
200-250	0.860	86.0
250-300	0.920	92.0
300-350	0.920	92.0
350-400	0.930	93.0

[a] 4 cm pathlength; H_2O reference

[b] influent concentration = 30 mg/L

TABLE A-19. RECOVERY OF SUCCINIMIDE IN WATER ADJUSTED TO pH 7.05 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	29.5	-
0-10	7.0	23.8
10-20	11.6	39.5
20-30	15.5	52.6
30-40	18.8	63.8
40-50	21.0	71.2
50-60	22.7	77.0
60-70	23.8	80.8
70-80	24.2	82.2
80-90	25.1	84.9
90-100	25.9	87.7
100-125	25.9	87.7
125-150	26.7	90.4
150-175	27.3	92.6
175-200	27.5	93.2
200-250	27.5	93.2
250-300	27.5	93.2
300-350	27.5	93.2
350-400	27.5	93.2

TABLE A-20. RECOVERY OF PURINE IN WATER ADJUSTED TO pH 2.0 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	absorbance at 260 nm (A.U.)[a]	% recovery
influent	0.550[b]	-
0-5	0.056	10.2
5-10	0.185	33.6
10-15	0.268	48.7
15-20	0.282	51.3
20-25	0.330	60.0
25-30	0.380	69.1
30-35	0.428	77.8
35-40	0.444	80.7
40-45	0.468	85.1
45-50	0.480	87.3
50-55	0.485	88.2
55-60	0.490	89.1
60-65	0.498	90.6
65-70	0.500	90.9
70-75	0.510	92.7
75-80	0.510	92.7
80-85	0.510	92.7
85-90	0.520	94.6
90-95	0.520	94.6
95-100	0.520	94.6
100-125	0.520	94.6
125-150	0.530	96.4
150-175	0.540	98.2
175-200	0.540	98.2
200-250	0.530	96.4
250-300	0.540	98.2

[a] 1 cm pathlength; H_2O reference

[b] influent concentration = 10 mg/L

TABLE A-21. RECOVERY OF PURINE IN WATER ADJUSTED TO pH 7.02 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	10.2	-
0-5	3.9	38.4
5-10	5.6	55.1
10-15	6.9	67.7
15-20	7.4	72.6
20-25	8.0	77.9
25-30	8.5	82.6
30-35	8.8	85.8
35-40	9.2	89.8
40-45	9.4	91.3
45-50	9.5	92.9
50-55	9.6	93.7
55-60	9.7	94.4
60-65	9.5	94.4
65-70	9.8	96.0
70-75	9.9	96.8
75-80	9.9	96.8
80-85	10.0	97.6
85-90	10.0	97.6
90-95	10.0	97.6
95-100	10.0	99.9
100-125	10.0	97.6
125-150	10.2	99.2
150-175	10.2	99.2
175-200	10.2	100
200-250	9.8	96.0
250-300	10.0	99.9
300-350	10.2	99.9
350-400	10.2	99.9

TABLE A-22. RECOVERY OF PYRIMIDINE IN WATER ADJUSTED TO pH 2.0 WITH HYDROCHLORIC ACID AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquots (ml)	absorbance at 242 nm (A.U.)[a]	% recovery
influent	0.465[b]	-
0-5	0.002	0.4
5-10	0.055	11.8
10-15	0.104	22.4
15-20	0.137	29.5
20-25	0.182	39.1
25-30	0.222	47.7
30-35	0.257	55.2
35-40	0.282	60.7
40-45	0.305	65.6
45-50	0.320	68.8
50-55	0.338	72.7
55-60	0.349	75.1
60-65	0.359	77.2
65-70	0.369	79.4
70-75	0.373	80.2
75-80	0.381	81.9
80-85	0.380	81.7
85-90	0.380	81.7
90-95	0.390	83.9
95-100	0.390	83.9
100-125	0.399	85.8
125-150	0.410	88.2
150-175	0.418	89.9
175-200	0.485	91.4
200-250	0.480	90.3
250-300	0.440	94.6
300-350	0.440	94.6
350-400	0.440	94.6

[a] 1 cm pathlength; H_2O reference

[b] influent concentration = 10 mg/L

TABLE A-23. RECOVERY OF PYRIMIDINE IN WATER ADJUSTED TO pH 7.00 WITH NaOH AND HCl AFTER PASSAGE THROUGH XAD-8 AND TENAX GC MACRORETICULAR RESINS

aliquot (ml)	concentration (mg/L)	% recovery
influent	9.8	-
0-5	0.6	5.6
5-10	1.8	18.3
10-15	3.3	34.1
15-20	4.4	45.3
20-25	5.4	55.0
25-30	6.0	61.5
30-35	6.7	68.8
35-40	7.4	74.5
40-45	7.8	79.8
45-50	8.1	83.1
50-55	8.4	86.1
55-60	8.7	89.0
60-65	8.9	91.1
65-70	9.0	92.4
70-75	9.1	93.7
75-80	9.2	94.7
80-85	9.3	95.4
85-90	9.4	96.0
90-95	9.4	96.0
95-100	9.4	96.7
100-125	9.4	96.7
125-150	9.6	98.0
150-175	9.5	97.6
175-200	9.6	98.4
200-250	9.5	97.6
250-300	9.6	98.7
300-350	9.7	99.0
350-400	9.7	99.3

TABLE A-24. [illegible] SQUARE EQUATIONS AN[illegible] VALU[illegible] [illegible]OR SELECTED NITROGENOUS COMPOUNDS DISSOLVED IN [illegible]ER (pH [illegible]ADJUSTED)

compound (λ: pathlength cm)	concentration mg/L	concentration M/L	[illegible]	molar extinction value	least square fit through origin[c]
uracil (233:4)	1.0	8.92×10^{-6}	.079	2.214×10^{3}	
	2.5	2.23×10^{-5}	.197	2.179×10^{3}	
	5.0	4.46×10^{-5}	.398	2.230×10^{3}	concentration (mg/L) = 125.5 x absorbance
	10.0	8.92×10^{-5}	.789	2.236×10^{3}	
				$(\overline{X} = 2.215 \times 10^{3})$	
				$(\sqrt{s^2} = 25.6)$	
uracil (254:1)	1.0	8.92×10^{-6}	.067	7.510×10^{3}	
	2.5	2.23×10^{-5}	.182	8.161×10^{3}	
	5.0	4.46×10^{-5}	.343	7.689×10^{3}	concentration (mg/L) = 144.4 x absorbance
	10.0	8.92×10^{-5}	.692	7.757×10^{3}	
				$(\overline{X} = 7.779 \times 10^{3})$	
				$(\sqrt{s^2} = 274.9)$	
creatine (210:4)	1.0	6.71×10^{-6}	.077	2.871×10^{3}	
	2.5	1.68×10^{-5}	.125	1.865×10^{3}	
	5.0	3.35×10^{-5}	.295	2.200×10^{3}	concentration (mg/L) = 15.2 x absorbance
	10.0	6.71×10^{-5}	.681	2.522×10^{3}	
				$(\overline{X} = 2.365 \times 10^{3})$	
				$(\sqrt{s^2} = 431.2)$	
creatine (233:4)	10.0	6.71×10^{-5}	.039	1.454×10^{2}	
creatine (254:4)	10.0	6.71×10^{-5}	.007	2.610×10^{1}	

(continued)

TABLE A-24 (continued)

compound (λ: pathlength cm)	concentration mg/L	concentration M/L	absorbance[a] (A.U.)	molar extinction value[b]	least square fit through origin[c]
cytosine (254:1)	1.0	9.0×10^{-6}	.010	1.111×10^{3}	concentration (mg/L) = 23.7 x absorbance
	2.0	1.8×10^{-5}	.072	4.000×10^{3}	
	4.0	3.6×10^{-5}	.161	4.472×10^{3}	
	5.0	4.5×10^{-5}	.191	4.244×10^{3}	
	10.0	9.0×10^{-5}	.458	5.089×10^{3}	
	20.0	1.8×10^{-4}	.830	4.611×10^{3}	
				$(\bar{X} = 3.921 \times 10^{3})$	
				$(\sqrt{s^2} = 1{,}424.8)$	
creatinine (254:1)	1.0	8.84×10^{-6}	.007	7.918×10^{2}	concentration (mg/L) = 76.2 x absorbance
	4.0	3.54×10^{-5}	.028	7.918×10^{2}	
	5.0	4.42×10^{-5}	.043	9.728×10^{2}	
	8.0	7.07×10^{-5}	.093	1.315×10^{3}	
	40.0	3.54×10^{-4}	.530	1.499×10^{3}	
				$(\bar{X} = 1.074 \times 10^{3})$	
				$(\sqrt{s^2} = 319.5)$	
indole (254:1)	1.0	8.54×10^{-5}	.036	4.217×10^{3}	concentration (mg/L) = 29.5 x absorbance
	4.0	3.42×10^{-5}	.125	3.661×10^{3}	
	5.0	4.27×10^{-5}	.155	3.631×10^{3}	
	10.0	8.54×10^{-5}	.334	3.912×10^{3}	
	11.7	9.99×10^{-5}	.410	4.105×10^{3}	
indole				$(\bar{X} = 3.905 \times 10^{3})$	
				$(\sqrt{s^2} = 260.8)$	

(continued)

TABLE A-24 (continued)

compound (λ: pathlength cm)	concentration mg/L	concentration M/L	absorbance[a] (A.U.)	molar extinction value[b]	least square fit through origin[c]
humic acid (330:1)	1.0		.027		
	4.0		.107		
	9.9		.236		concentration (mg/L) = 41.9 x absorbance
	19.8		.470		
tryptophan (278:1)	1	4.896×10^{-6}	.034	6.944×10^{3}	
	2	9.793×10^{-6}	.062	6.331×10^{3}	
	3	1.469×10^{-5}	.101	6.875×10^{3}	
	5	2.448×10^{-5}	.162	6.618×10^{3}	concentration (mg/L) = 32.9 x absorbance
	10	4.896×10^{-5}	.295	6.025×10^{3}	
				($\overline{X} = 6.559 \times 10^{3}$)	
				($\sqrt{s^2} = 383.6$)	
adenine (256:1)	1	7.400×10^{-6}	.105	1.419×10^{4}	
	2	1.480×10^{-5}	.209	1.412×10^{4}	
	3	2.220×10^{-5}	.306	1.378×10^{4}	concentration (mg/L) = 9.9 x absorbance
	5	3.700×10^{-5}	.510	1.378×10^{4}	
	10	7.400×10^{-5}	1.000	1.351×10^{4}	
				($\overline{X} = 1.388 \times 10^{4}$)	
				($\sqrt{s^2} = 278.6$)	
succinimide (215:4)	1	1.009×10^{-5}	0.013	3.220×10^{2}	
	2	2.018×10^{-5}	0.034	4.213×10^{2}	
succinimide (215:4)	3	3.028×10^{-5}	0.045	3.715×10^{2}	
	5	5.046×10^{-5}	0.058	2.873×10^{2}	concentration (mg/L) = 80.8 x absorbance
	10	1.009×10^{-4}	0.120	2.973×10^{2}	
				($\overline{X} = 3.399 \times 10^{2}$)	
				($\sqrt{s^2} = 56.0$)	

(continued)

TABLE A-24 (continued)

compound (λ: pathlength cm)	concentration mg/L	concentration M/L	absorbance[a] (A.U.)	molar extinction value[b]	least square fit through origin[c]
5-chlorouracil (272:1)	1	6.826×10^{-6}	0.059	8.644×10^{3}	
	2	1.365×10^{-5}	0.117	8.570×10^{3}	
	3	2.048×10^{-5}	0.164	8.009×10^{3}	
	5	3.413×10^{-5}	0.269	7.882×10^{3}	concentration (mg/L) = 18.6 x absorbance
	10	6.862×10^{-4}	0.533	7.808×10^{2}	
				$(\bar{X} = 8.183 \times 10^{3})$	
				$(\sqrt{s^2} = 394.9)$	
pyrimidine (242:1)	1	1.249×10^{-5}	.031	2.483×10^{3}	
	2	2.497×10^{-5}	.066	2.643×10^{3}	
	3	3.746×10^{-5}	.103	2.750×10^{3}	
	5	6.243×10^{-5}	0.156	2.499×10^{3}	concentration (mg/L) = 32.3 x absorbance
	10	1.249×10^{-4}	0.305	2.443×10^{3}	
				$(\bar{X} = 2.564 \times 10^{3})$	
				$(\sqrt{s^2} = 128.7)$	
purine (260:1)	1	8.325×10^{-6}	0.064	7.688×10^{3}	
	2	1.665×10^{-5}	0.126	7.568×10^{3}	
	3	2.498×10^{-5}	0.197	7.888×10^{3}	concentration (mg/L) = 16.1 x absorbance
	5	4.163×10^{-5}	0.311	7.471×10^{3}	
purine (260:1)	10	8.235×10^{-5}	0.616	7.399×10^{3}	
				$(\bar{X} = 7.6028 \times 10^{3})$	
				$(\sqrt{s^2} = 192.8)$	

[a] pathlength = 1 cm; absorbance read against water reference

[b] $E = A(C\ell)^{-1}$ where A = absorbance; C = molar concentration; E = molar extinction value;
ℓ = pathlength = 1 cm

[c] proportionality constant = $\Sigma AC/\Sigma A^2$ (least squares equation constrained to pass through origin)

TABLE A-25. PERCENT RECOVERY OF MIXTURE OF NITROGENOUS COMPOUNDS AFTER PASSAGE THROUGH XAD-8 AND TENAX RESINS (pH OF MIXTURE UNADJUSTED)

compound	15-20 ml		20-45 ml		45-100 ml		100-150 ml		150-200 ml		200-300 ml		300-400 ml	
	A[a]	H[b]	A	H	A	H	A	H	A	H	A	H	A	H
uracil	35.5	63.6	59.7	81.8	93.5	98.9	88.7	99.4	93.5	100	100	100	82.3	93.2
indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tyrosine	17.1	16.7	55.7	60.4	70.0	83.3	67.1	85.4	82.9	93.8	88.6	100	67.1	93.8
purine	29.9	16.7	44.4	41.7	99.1	70.8	81.2	83.3	100	91.7	100	100	94.0	95.8
guanine	20.2	7.8	29.6	39.7	40.5	65.5	52.6	78.4	48.6	84.5	55.5	91.4	53.0	86.2
cytosine	18.4	19.4	23.3	25.0	77.9	74.1	88.3	93.5	98.8	97.2	100	100	89.9	100
adenine	37.8	20.8	14.1	11.5	58.4	57.3	77.0	78.1	86.5	95.8	98.9	100	69.2	93.8
creatinine	33.1	25.3	11.1	13.3	67.8	63.9	89.2	85.5	98.5	96.4	100	100	56.0	94.0
tryptophan	0	0	0	0	97.9	75.0	65.1	75.0	17.5	58.3	100	69.4	73.2	83.3

[a] A = peak area computed from integrator counts

[b] H = peak height

TABLE A-26. PERCENT RECOVERY OF MIXTURE OF NITROGENOUS COMPOUNDS DISSOLVED IN A SOLUTION CONTAINING 20 mg/L HUMIC ACID[c] ACIDIFIED TO pH 2.0 AFTER PASSAGE THROUGH XAD-8 AND TENAX RESINS

Compound	30-40 ml		40-50 ml		50-75 ml		75-100 ml		100-150 ml		150-200 ml		200-300 ml		300-400 ml		400-500 ml	
	A[a]	H[b]	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H
uracil	25.7	40.0	34.3	46.7	77.1	74.7	65.7	77.3	77.1	84.0	94.3	93.3	91.4	82.7	100	100	94.3	81.3
indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tyrosine	0	0	0	0	60.0	50.0	67.5	63.3	84.2	47.5	100	100	53.3	87.4	100	100	62.9	81.3
purine and guanine	12.6	12.5	39.3	38.5	74.8	74.0	80.8	82.3	86.7	78.1	99.1	94.8	85.0	86.5	100	100	82.3	80.2
cytosine	11.0	8.0	43.7	34.0	90.4	70.0	81.5	72.1	92.7	74.0	85.9	90.0	90.4	76.0	100	100	79.3	74.0
adenine	7.8	7.8	29.5	33.3	61.1	68.6	70.5	72.5	76.3	74.5	96.9	92.2	72.5	78.4	100	100	40.4	74.5
creatinine	-	12.5	-	29.2	69.2	75.0	70.9	100	22.9	100	92.3	95.8	65.4	75.0	100	100	77.8	88.7
tryptophan	0	0	0	0	0	0	0	0		9.4	74.7	62.5	-	100	34.9	100	100	62.5

[a] A = peak area as computed from integrator counts

[b] H = peak height

[c] humic acid not eluted

TABLE A-27. VALUES OF KJELDAHL-N BLANKS (mg/L)[a]

digestion solution composition	sample	reference cell	digestion duration (minutes)	absorbance at 635 nm
A. 134 g K_2SO_4	reagent	undigested	60	.075
5 ml $SeOCl_2$	blank	NH_3-N	120	.082
200 ml H_2SO_4		blank	180	.068
diluted to				
1 liter				
B. 20 g K_2SO_4	reagent	undigested	120	.086
0.2 g SeO_2	blank	NH_3-N	180	.072
110 ml H_2SO_4		blank		
diluted to				
1 liter				
C. 134 g K_2SO_4	reagent	undigested	60	.081
2 g HgO	blank	NH_3-N	120	.052
200 ml H_2SO_4		blank	180	.044
diluted to				
1 liter				
D. 20 g K_2SO_4	reagent	distilled	120	.140
0.1 g SeO_2	blank	water		.136
500 ml H_2SO_4				.147
diluted to				.152
1 liter				.172
				(mean = 0.149)
				(standard deviation = 0.014)

[a] determined by Scheiner's method (79)

TABLE A-28. EFFICIENCIES OF DIGESTION COMPOSITION AND DURATION

source	sample	digestion duration (minutes)	Kjeldahl-N [a] (mg/L)	digestion solution composition
Charles River (12/19/77)	XAD-8 filtered	60	4.2	134 g K_2SO_4 5 ml $SeOCl_2$ 200 ml H_2SO_4 diluted to liter with H_2O
		60	4.0	
		120	4.1	
		120	4.0	
		180	4.0	
		180	3.9	
Charles River (12/19/77)	XAD-8 filtered	180	4.0	20 g K_2SO_4; 0.2 g SeO_2 110 ml H_2SO_4 diluted to 1 liter with water
		180	3.8	
Charles River (12/16/77)	filtered to 10 µm	70	7.0	134 g K_2SO_4; 4 ml $SeOCl_2$ 200 ml H_2SO_4 diluted to 1 liter with water
		150	7.0	
Charles River (12/16/77)	XAD-8 filtered	70	7.1	
Charles River (12/16/77)	filtered to 10 µm	180	7.8	134 g K_2SO_4; 2 g HgO 200 ml H_2SO_4 diluted to 1 liter with water
Charles River (12/16/77)	XAD-8 filtered	180	2.4	

[a] determined by Scheiner's method (79).

TABLE A-29. RETENTION TIMES (IN MINUTES) OF NITROGENOUS ORGANIC COMPOUNDS

0-1	1-3	6-8	12-13	19-23
creatine	caffeine	purine	cytosine	adenine
thymine	tryptophan	guanine		
uracil	indole			
pyridine				
pyrrole				
creatinine				
uric acid				
hydroxyproline				
aspartic acid				
succinimide				
alanine				
barbituric acid				
hydroxyproline				
ℓ-(-)histidine				

column: Zipax SCX
mobile phase: 0.01 M HNO_3
flow rate: 1.2 ml/min
wavelength: 254 nm
temperature: 30°C

TABLE A-30. RETENTION TIMES (IN MINUTES) OF NITROGENOUS ORGANIC COMPOUNDS

1 - 1.5	1.5 - 2	2 - 2.3	2.3 - 2.5
cytosine	uracil	pyrrole	adenine
pyridine	proline	thymine	purine
		creatine	guanine
		ℓ-(-)histidine	
		creatinine	
		uric acid	
		succinimide	
		aspartic acid	
		alanine	
		hydroxyproline	
		tryptophan	
		indole	

column: Aminex A-27
mobile phase: 0.325 M ammonium acetate
flow rate: 0.5 ml/min
wavelength: 254 nm
temperature: 30°C

TABLE A-31. AVERAGE ELUTION POSITIONS (ml) OF NITROGENOUS COMPOUNDS USING DIFFERENT CHROMATOGRAPHIC COLUMNS AND MOBILE PHASES

column	mobile phase	cytosine	creatinine	alanine	indole	water	resolution[a]
Zipax SCX	10^{-3} M perchloric				2.0	1.37	
Zipax SCX	10^{-4} M perchloric				2.0	1.52	
Zipax SCX	10^{-3} M H_3PO_4	N.obs[b]			1.91	N.obs	
Zipax SCX	0.172 M HAc (pH=2.5)				1.87	1.54	
Zipax SCX	0.05 M H_3PO_4 (pH=2.1)	42 & 55[c]		7.55	1.96	1.51	1/1/1
Zipax SCX	10^{-2} HNO_3 (pH=2.0)	10.3 & 24	8.6 & 34	4.84	2.0	1.59	1/1/1/1
Zipax SCX	10^{-2} HNO_3	46	N.obs	6.6	1.77	1 & 1.5	1/1/1
Zipax SCX	10^{-2} HNO_3	39	29 & 41	6.6	1.8	1.5	1/1/2
Zipax SCX	10^{-2} HNO_3	36	27 & 43	6.2	1.9	1.5	1/1/2
Zipax SCX	0.7 x 10^{-2} HNO_3	50.5	30 & 46				poor
Zipax SCX	0.3 x 10^{-2} HNO_3	N.obs	N.obs				
Zipax SCX	0.5 x 10^{-2} HNO_3	N.obs					
Zipax SCX	0.6 x 10^{-2} HNO_3	72.5	75 & 115				poor
Zipax SCX	10^{-2} HNO_3 with 1% acetonitrile	37.8	35 & 58				1/1
Aminex A-27	3 M NH_4Ac/HAc					1 & 1.4	
Aminex A-27	0.3 M NH_4Ac/HAc					0.8 & 4.0	
Zipax SCX	0.5 M $NaNO_3$, 4 x 10^{-3} HNO_3	21.1	N.obs	3.74	2.02	1.48	
Zipax SCX	10^{-2} HNO_3	25.3	14.5	5.09	1.88	1.55	1/1/1/1

[a] resolution: numbers indicate quantity of compounds each successive chromatographic peak (not including water peak).

[b] N.obs = no peak observed.

[c] Two values indicate more than one peak observed for the compound.

TABLE A-32. RETENTION VALUES (ml) OF SELECTED NITROGENOUS COMPOUNDS ON ZORBAX CN

mobile phase	cytosine	creatinine	alanine	indole	water	resolution[a]
.05 M NaAc/HAc buffer (pH 5.0); 29% MeOH	3.25	3.29	13.3	46.8	2.5 & 5.0	2/1/1
.05 M NaAc/HAc buffer (pH 5.0); 15% acetonitrile	3.42	3.3	19.8	29.9	2.2 & 5.0	2/1/1
.05 M NaAc/HAc buffer (pH 5.0); 1% acetonitrile	3.78	3.86	N. obs[b]	87.54	2.8 & 3.7	2/1
.05 M NaAc/HAc buffer (pH 5.0); 30% acetonitrile	16.2	17.4	N. obs	14.6 & 18.8	3.2 & 3.3	1/1/1
.005 M NaAc/HAc buffer (pH 5.0); 40% acetonitrile	14.7	10.4	-	20.64	2.6 & 3.4	2/1
.05 M NaAc/HAc buffer (pH 5.0); 40% acetonitrile	2.89	2.89	-	10.8	2.3 & 3.1	2/1
.05 M NaAc; .05 N HAc; (pH 4.74); 25% acetonitrile	2.85	3.01	-	24.6	-	2/1
.05 M NaAc; .025 N HAc (pH 5.0)	4.07	4.16	-	103.67	-	2/1
.01 M phosphate buffer (pH 6.9)	4.26	4.16	-	96.0	-	2/1

[a] resolution: numbers indicate quantity of compounds comprising each successive chromatographic peak (not including water peaks).

[b] N.obs = no peaks observed.

TABLE A-33. RETENTION VALUES (ml) OF SELECTED NITROGENOUS COMPOUNDS ON ZORBAX C8

mobile phase	cytosine	creatinine	alanine, uracil & guanine	indole	resolution[a]
.05 M NaAc/HAc buffer (pH 5.0); 30% acetonitrile (1/26/78)	2.64	2.67	-	42.5	2/1
.01 M phosphate buffer[b] (pH 6.9); 1/27/78	2.35	2.35	-	-	unresolved
.001 M phosphate buffer[b] (pH 6.9)	2.22	2.23	-	-	unresolved
.01 M phosphate buffer (pH 7.0); KH_2PO_4 + NaOH	2.66	2.71	-	-	unresolved
.05 M phosphate buffer (pH 6.9); 30% acetonitrile 1/27/78	-	-	-	35.96	-
.05 N NaAc/HAc buffer (pH 5.0); 15% acetonitrile	3.08	3.08	2.6	158.0	3/1

[a] resolution: numbers indicate quantity of compounds comprising each successive chromatographic peak (not including water peaks)

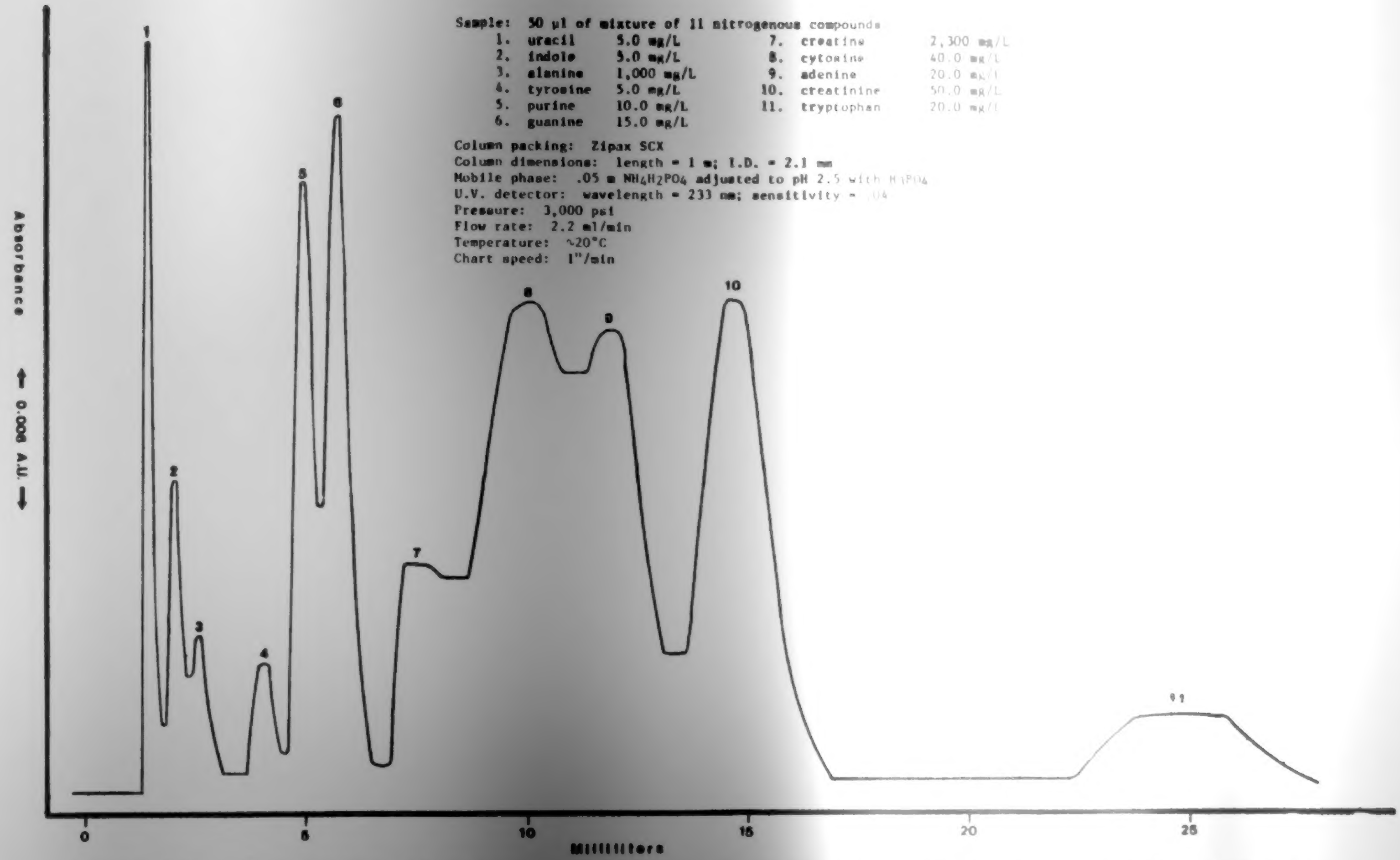
[b] column temperature heated to 33°C

TABLE A-34. RETENTION VOLUMES OF CREATINE ON ZIPAX SCX[a]

concentration (mg/L)	retention (mL)
100	10.7
200	9.9
300	10.2
500	9.2
1,000	8.5
2,000	7.9
2,300	7.6

[a] mobile phase: .05 M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4

APPENDIX B

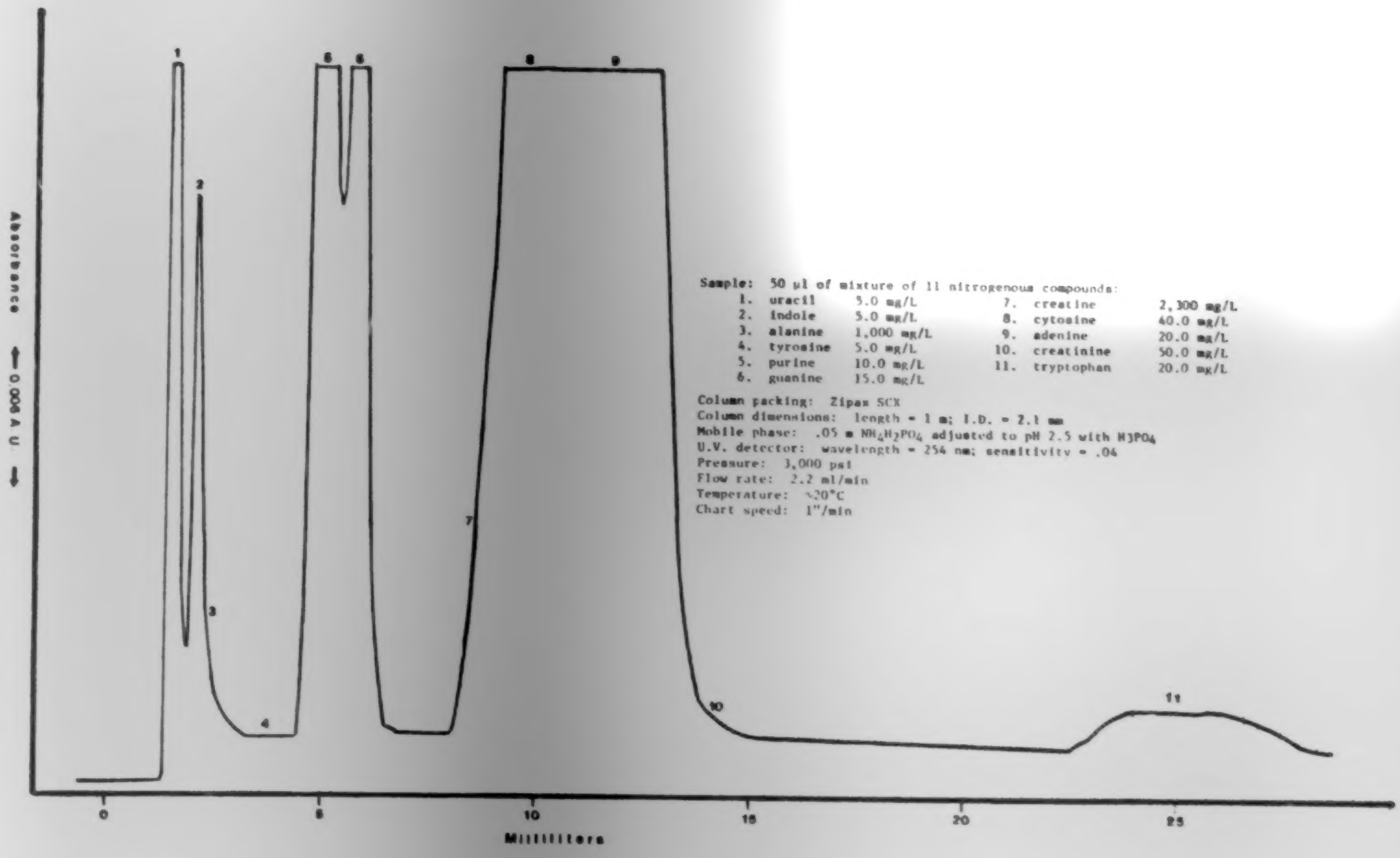


Sample: 50 µl of mixture of 11 nitrogenous compounds

1.	uracil	5.0 mg/L	7.	creatine	2,300 mg/L
2.	indole	5.0 mg/L	8.	cytosine	40.0 mg/L
3.	alanine	1,000 mg/L	9.	adenine	20.0 mg/L
4.	tyrosine	5.0 mg/L	10.	creatinine	50.0 mg/L
5.	purine	10.0 mg/L	11.	tryptophan	20.0 mg/L
6.	guanine	15.0 mg/L			

Column packing: Zipax SCX
Column dimensions: length = 1 m; I.D. = 2.1 mm
Mobile phase: .05 m $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4
U.V. detector: wavelength = 233 nm; sensitivity = .04
Pressure: 3,000 psi
Flow rate: 2.2 ml/min
Temperature: ~20°C
Chart speed: 1"/min

Figure B-1. Chromatogram of mixture of 11 nitrogenous compounds resolved on Zipax SCX (233 nm).



Sample: 50 µl of mixture of 11 nitrogenous compounds:

1.	uracil	5.0 mg/L	7.	creatine	2,300 mg/L
2.	indole	5.0 mg/L	8.	cytosine	40.0 mg/L
3.	alanine	1,000 mg/L	9.	adenine	20.0 mg/L
4.	tyrosine	5.0 mg/L	10.	creatinine	50.0 mg/L
5.	purine	10.0 mg/L	11.	tryptophan	20.0 mg/L
6.	guanine	15.0 mg/L			

Column packing: Zipax SCX
Column dimensions: length = 1 m; I.D. = 2.1 mm
Mobile phase: .05 m $NH_4H_2PO_4$ adjusted to pH 2.5 with $H3PO_4$
U.V. detector: wavelength = 254 nm; sensitivity = .04
Pressure: 3,000 psi
Flow rate: 2.2 ml/min
Temperature: ∿20°C
Chart speed: 1"/min

Figure B-2. Chromatogram of mixture of 11 compounds resolved on Zipax SCX (254 nm).

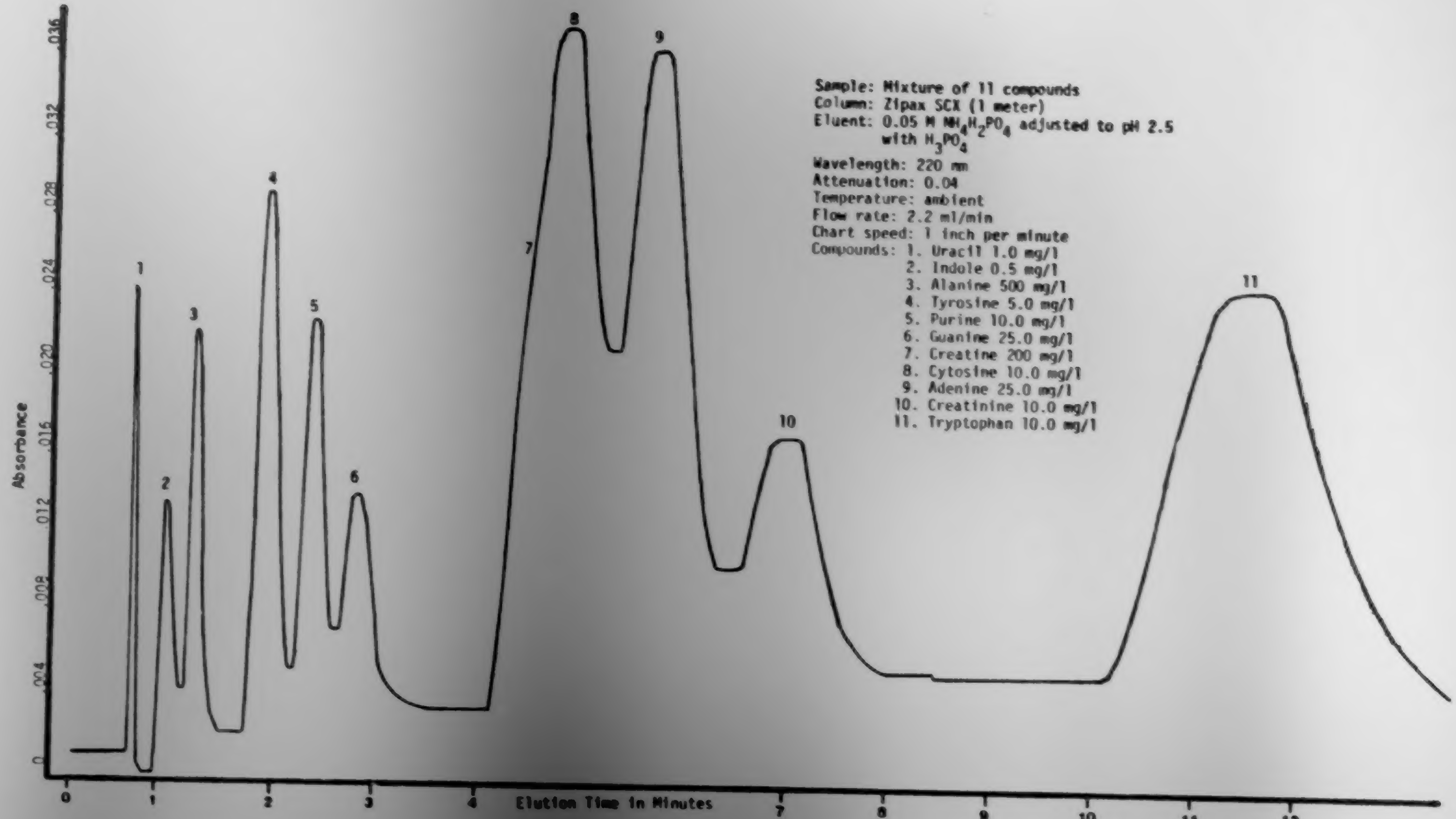


Figure B-3. Chromatogram of mixture of 11 nitrogenous compounds resolved on Zipax SCX (220 nm).

Reproduced from best available copy.

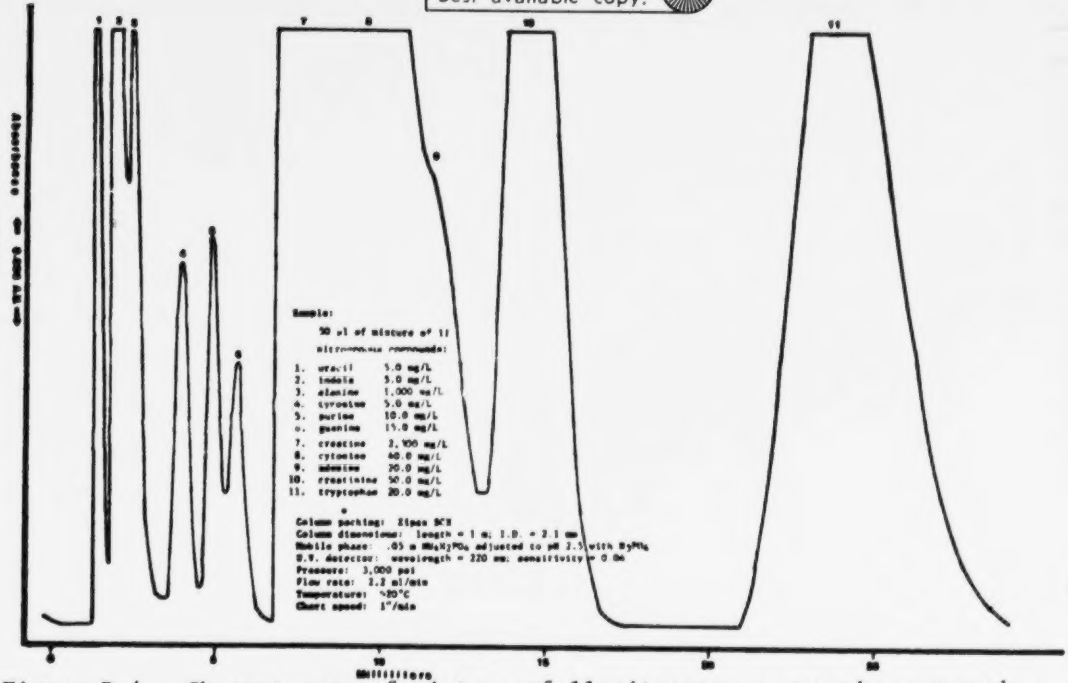


Figure B-4. Chromatogram of mixture of 11 nitrogenous organic compounds resolved on Zipax SCX (220 nm).

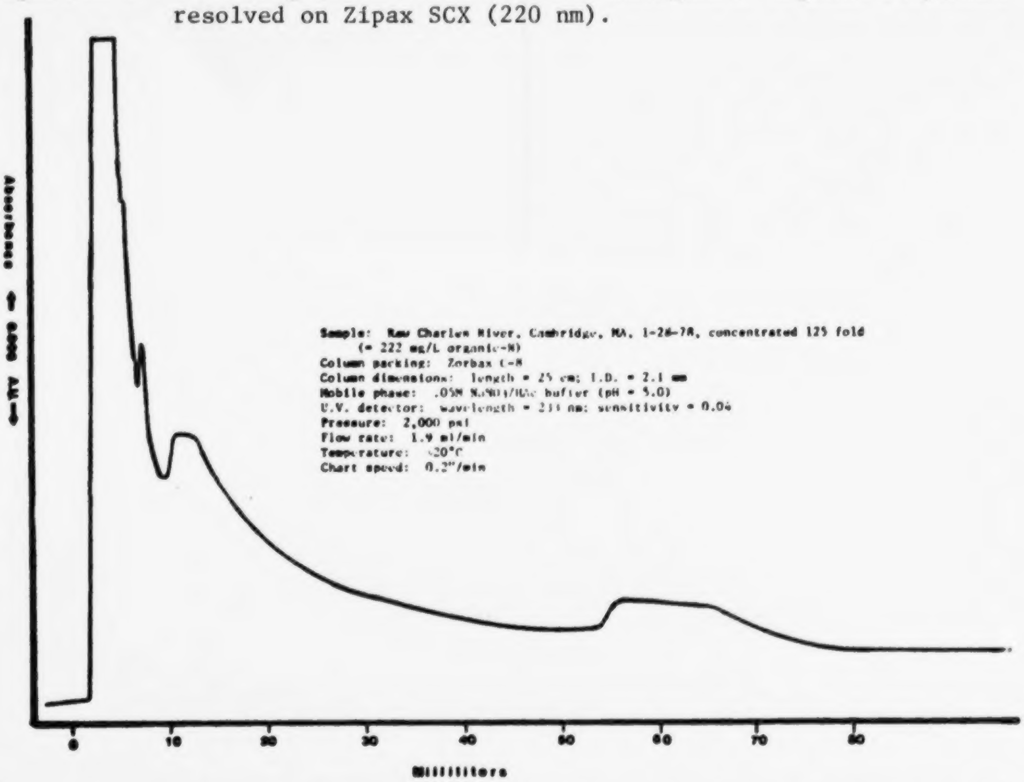
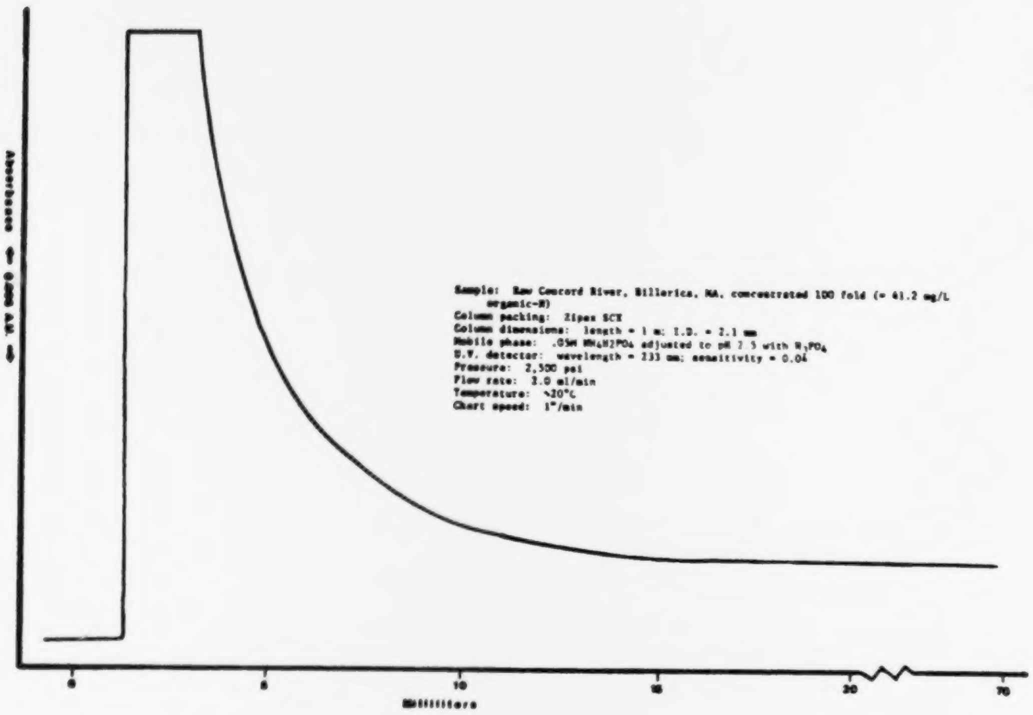
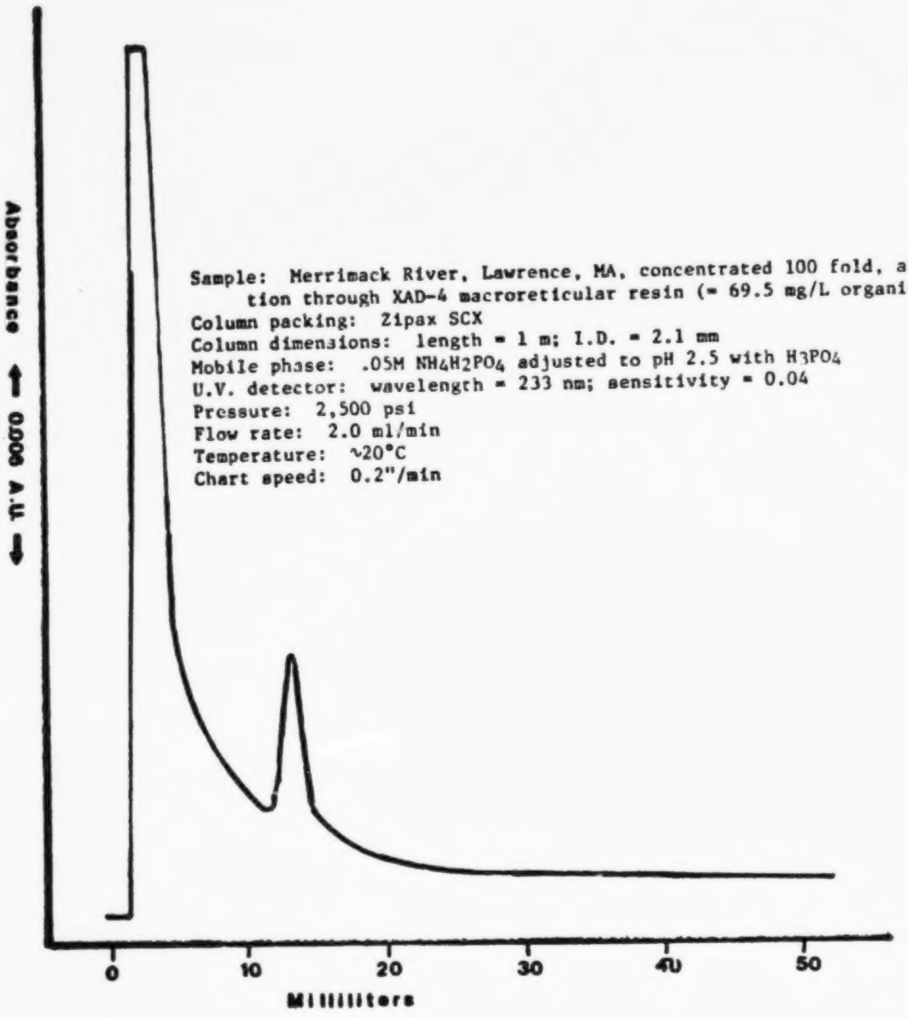


Figure B-5. Chromatogram of Charles River water sample, resolved on Zorbax C-8.



Sample: Raw Concord River, Billerica, MA, concentrated 100 fold (= 41.2 mg/L organic-N)
Column packing: Zipax SCX
Column dimensions: length = 1 m; I.D. = 2.1 mm
Mobile phase: .05M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4
U.V. detector: wavelength = 233 nm; sensitivity = 0.04
Pressure: 2,500 psi
Flow rate: 2.0 ml/min
Temperature: ~20°C
Chart speed: 1"/min

Figure B-6. Chromatogram of Concord River, Billerica, MA, resolved on Zipax SCX.



Sample: Merrimack River, Lawrence, MA, concentrated 100 fold, after filtration through XAD-4 macroreticular resin (= 69.5 mg/L organic-N).
Column packing: Zipax SCX
Column dimensions: length = 1 m; I.D. = 2.1 mm
Mobile phase: .05M $NH_4H_2PO_4$ adjusted to pH 2.5 with H_3PO_4
U.V. detector: wavelength = 233 nm; sensitivity = 0.04
Pressure: 2,500 psi
Flow rate: 2.0 ml/min
Temperature: ~20°C
Chart speed: 0.2"/min

Figure B-7. Chromatogram of Merrimack River water sample, resolved on Zipax SCX.

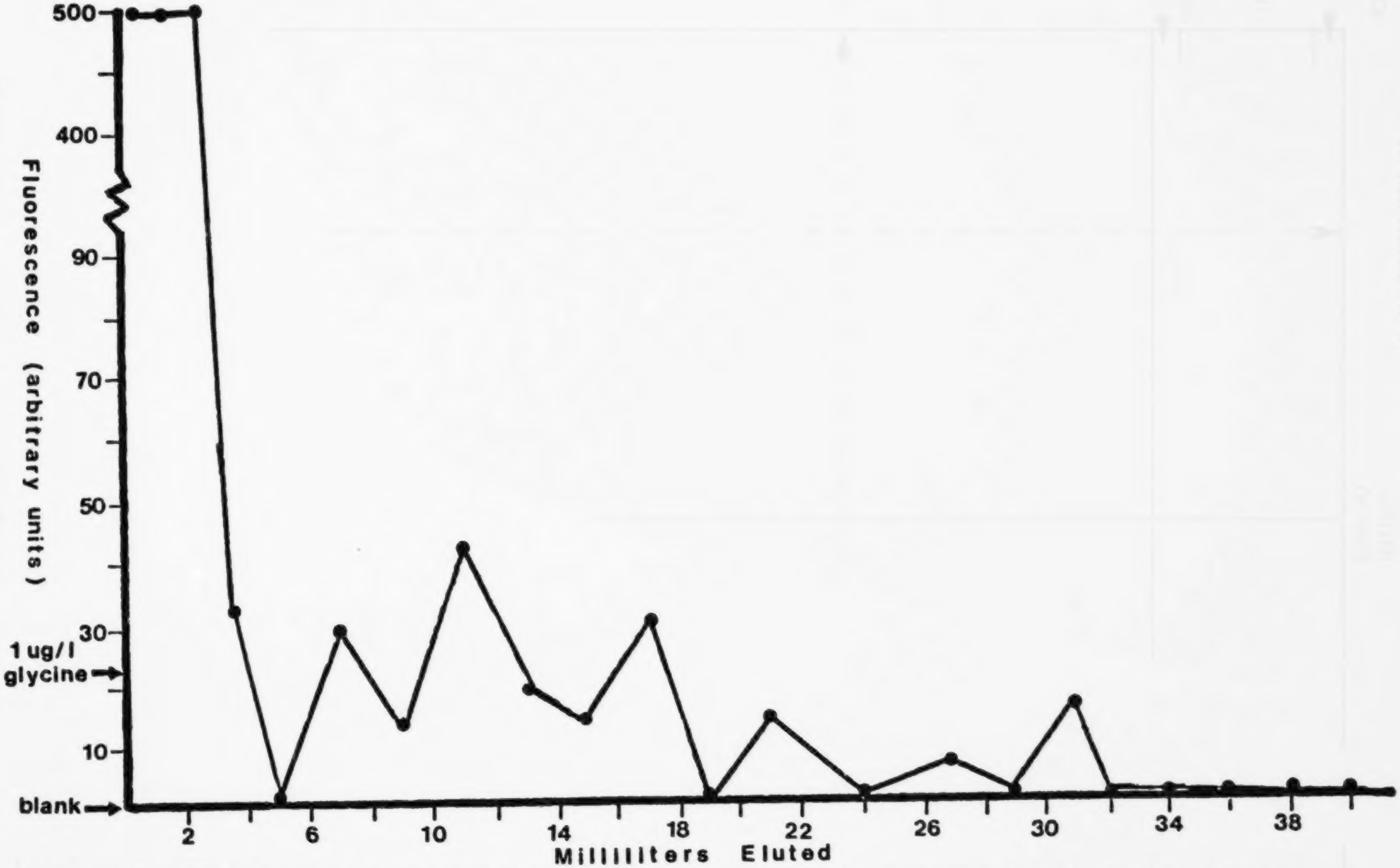


Figure B-8. Fluorescence of aliquots from the chromatographic column after injection of 50 µl concentrated Marlboro West, raw, prechlorinated sewage effluent. column: Zipax SCX. Mobile phase: .05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4.

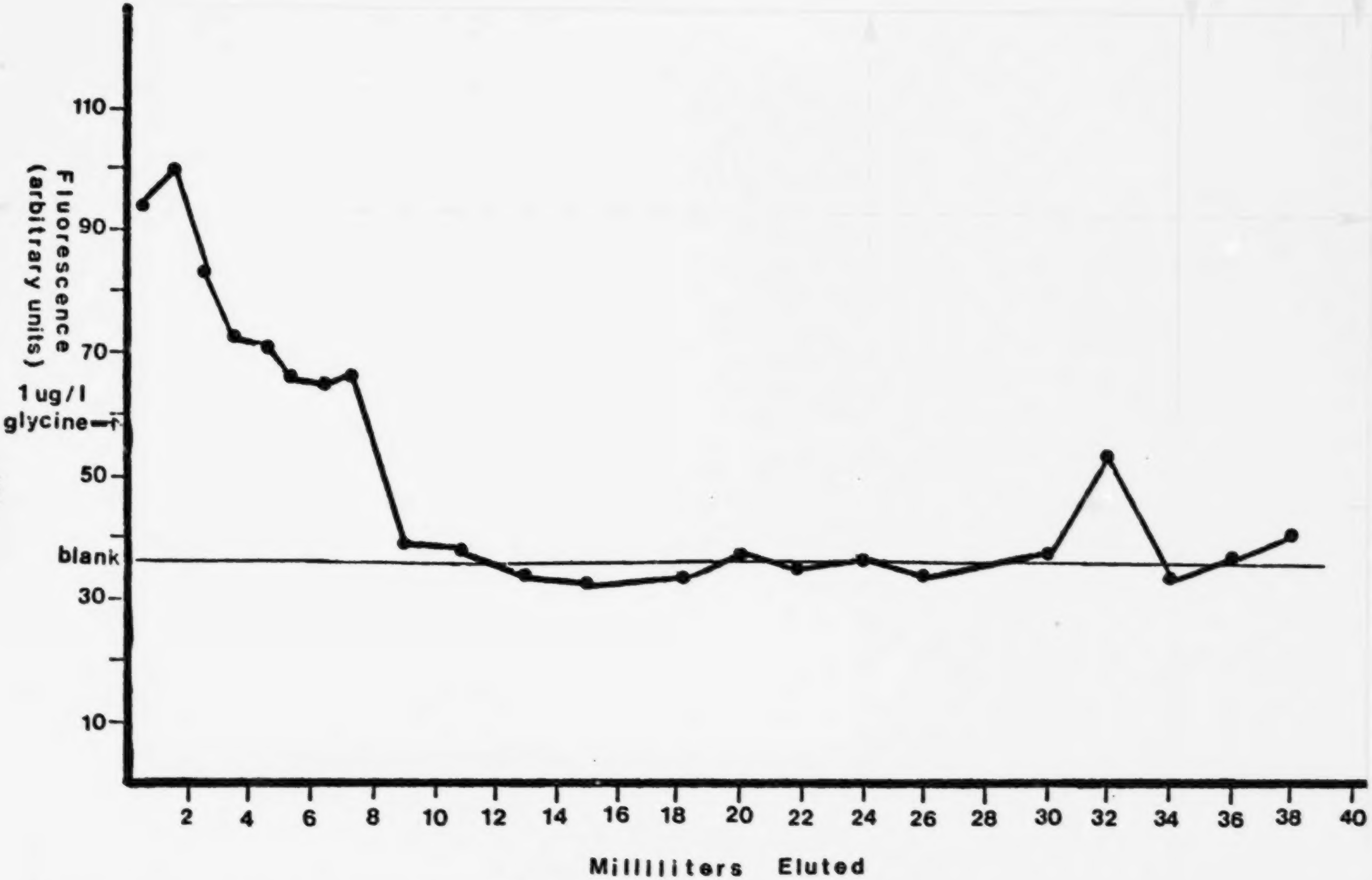
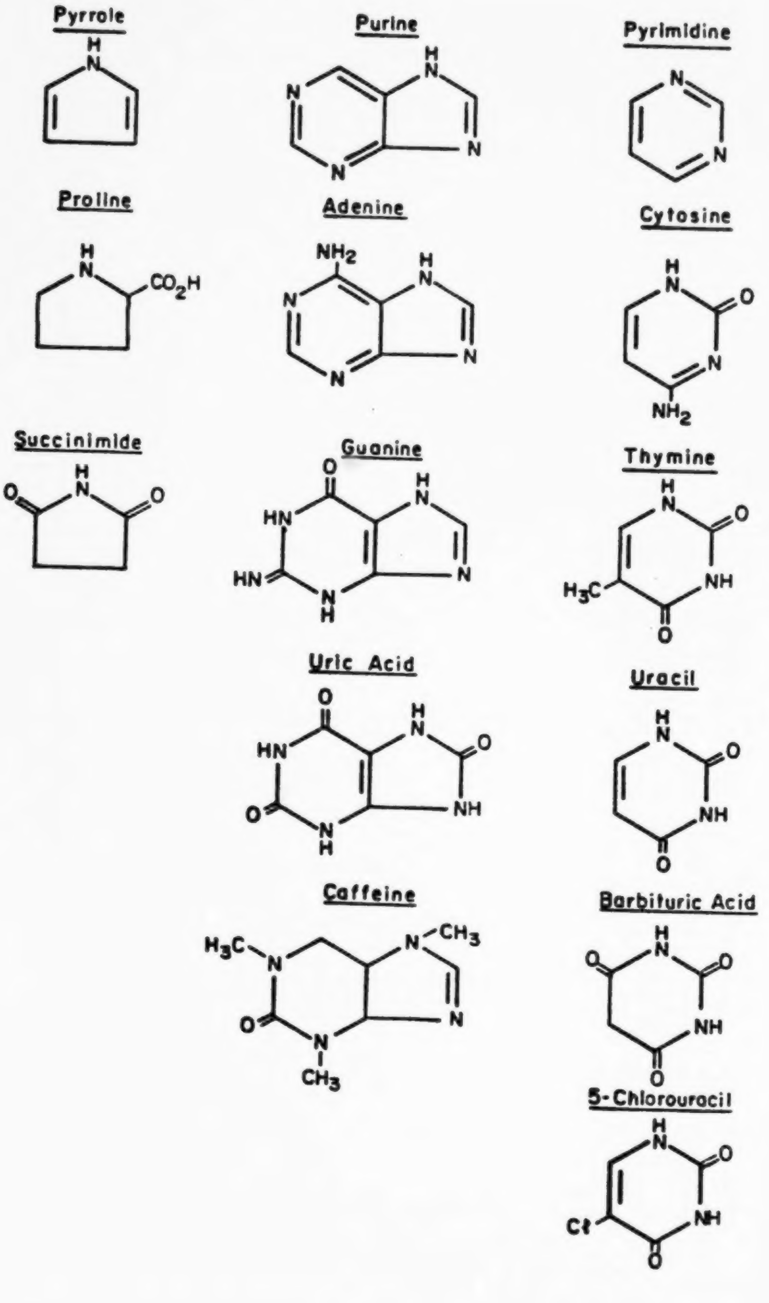


Figure B-9.. Fluorescence of aliquots from the chromatographic column after injection of 50 µl concentrated Marlboro West, raw, post chlorinated sewage effluent. Column: Zipax SCX. Mobile phase: 0.05 M NaH_2PO_4 adjusted to pH 2.5 with H_3PO_4.



Pyrrole

Purine

Pyrimidine

Proline

Adenine

Cytosine

Succinimide

Guanine

Thymine

Uric Acid

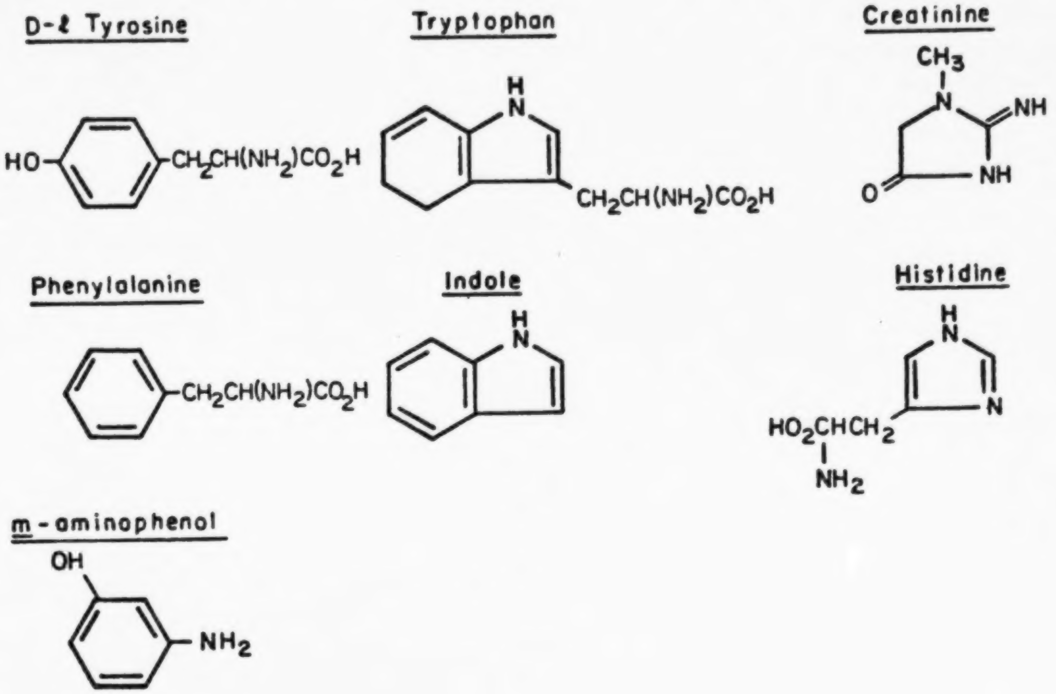
Uracil

Caffeine

Barbituric Acid

5-Chlorouracil

Figure B-10. Chemical structure of some nitrogenous organic compounds.



D-ℓ Tyrosine

Tryptophan

Creatinine

Phenylalanine

Indole

Histidine

m-aminophenol

HO
$CH_2CH(NH_2)CO_2H$
H
N
$CH_2CH(NH_2)CO_2H$
CH_3
N
NH
O
NH
$CH_2CH(NH_2)CO_2H$
H
N
H
N
N
HO_2CHCH_2
NH_2
OH
NH_2

Figure B-10. (continued)

APPENDIX C

LITERATURE REVIEW

THE HAZARDS OF CONSUMING CHEMICALLY CONTAMINATED DRINKING WATER

The proliferation of synthetic chemicals resulting from our expanding industrialized economy has led inevitably to the entry of organic compounds into our nation's water resources. Growing awareness of the deleterious effect of these trace contaminants on human health and the increasing number of organic chemicals identified in municipal water supplies have resulted in a nationwide effort to identify and quantify the full spectrum of organic compounds reaching the water consumer. Hundreds of organic contaminants, present at nanogram to parts per million concentrations, have now been identified in municipal water supplies (1). Improved analytical techniques have increased the number of organic chemicals identified in drinking water from just 10 in 1970 (2) to 300 in 1976 (3) and more than 700 specific compounds in 1978 (4). These values represent the total number of compounds identified for all communities investigated. The actual number of contaminants present in a single water supply is probably much smaller. The National Cancer Institute, working collaboratively with the U.S. Environmental Protection Agency, recently compiled a list of over 1,700 organic compounds found in various kinds of waters ranging from industrial effluents to drinking water (5-8).

The direct adverse effects of these compounds on the consumer include: 1) decreased aesthetic quality resulting from taste and odors, color, and foaming; 2) toxicological hazards, both acute and chronic; and 3) increased carcinogenic; or 4) mutagenic risks resulting from long-term, low-level exposure. Additional harmful compounds are produced indirectly by reaction of organic compounds with inorganic constituents.

Rook (9), and Bellar, Lichtenberg and Kroner (10) found that chloroform and other halogenated methanes are formed during the chlorination of water for disinfection. The carcinogenicity of these chlorinated products has caused concern about their persistence in the environment and the extent of their formation.

Unfortunately very little information is available about the human health effects of trace quantities of organic chemicals in water. Epidemiological studies on the effects of organic compounds identified in drinking water on animal or bacterial populations have been minimal (11). Heuper and Payne (12) demonstrated the formation of spindle cell sarcomas at the site of subcutaneous injection of carbon chloroform extracts (CCEO as well as internal papilloma and neoplastic reactions in mice. Jolley *et al.* (13) reported that organic residues isolated from drinking water by reverse osmosis produced mutagenesis in *Salmonella*. A number of unpublished studies reported by the director of the National Cancer Institute (14) have shown a pattern of statistical association between elevated cancer risk rates and surrogates for organic contaminants in drinking water.

Toxicological data on the risks of ingesting chemical pollutants in drinking water are also scarce. Tardiff (15) reported that 128 slightly to super toxic compounds and 43 suspect or positive carcinogens were present among the organic compounds listed as having been found in tap water. Kopfler (16) cited chloroethers and chlorobenzenes as examples of compounds identified in drinking water that may be responsible for some forms of chronic illness. The Environmental Protection Agency recently commissioned the Medical College of Virginia to undertake a two year study on the impact of trace organic compounds on human health. Although the leader of the research team was reported to have asserted that none of the 700 trace chemicals studied posed any significant threat to human health (17-19), this was later refuted by both the research chairman (20) and EPA (11). Findings of a 1977 National Academy of Sciences study on the effects of potential toxicants (11) listed one compound, vinyl chloride, as a known human carcinogen, and two (benzene and benzo (a)-pyrene) as suspected human carcinogens. Nineteen other compounds were listed as animal or suspected animal carcinogens. The study also presented acceptable daily intake (ADI) levels for 45 other organic compounds. ADI values indicate the level at which exposure to a single chemical is not anticipated to produce an observable toxic response in humans. The health effects of the majority of the 74 non-pesticides and 309 volatile organic compounds identified in drinking water and selected for review, could not be assessed because of inadequate or unavailable toxicological information.

The effects of chronic exposure to low does of organic micropollutants remain largely unknown. However, EPA's scientific advisory board (21) agreed that a currently unquantifiable human health risk exists from consumption of organically polluted drinking water. The bases for the opinion that there are hazards from consumption of chemically contaminated drinking water were summarized by the director of the National Cancer Institute as follows:

"1. Chemicals which have been shown to cause cancers in animal studies are commonly found in drinking water in small amounts.

2. Some known human carcinogens have been found in drinking water.

3. Exposure to even very small amounts of carcinogenic chemicals poses some risk and repeated exposures amplify the risk.

4. Cancers induced by exposure to small amounts of chemicals may not be manifested for 20 or more years and thus are difficult to relate to a single specific cause.

5. Some portion of the population that is exposed is at greater risk because of other contributed factors such as prior disease states, exposure to other chemicals, or genetic susceptibility (14)."

The Interim Primary Drinking Water Standards promulgated in December, 1974 and effective June 1977 (22) reflected the insufficiency of epidemiological and toxicological data on the health effects of organic chemicals by their exclusion of maximum contaminant levels (MCL) for specific organic chemicals other than certain pesticides. The original regulations did, however call for a maximum contaminant level for the total concentration of organic compounds, as measured by the carbon chloroform extract which was later withdrawn (21). A recent proposed amendment to the National Interim Primary Drinking Water Regulations (4) contained a maximum contaminant level of 0.1 mg/L for total trihalomethanes (TTHM's) including chloroform.

ORGANIC IMPURITIES IN NATURAL WATERS

Organic substances occur in the environment: either as the result of natural processes or their introduction by man. Natural sources contribute the majority of organic material in natural waters (4,23), via decay of vegetation and animal tissues (humic matter), animal excretion, photosynthetic byproducts and extracellular release of organic matter by plankton and aquatic macrophytes. Sources of man-derived organic contaminants include: domestic, agricultural and industrial wastes, accidental spillages, dispersed pesticides, rainfall, seepage and non-point-source runoff.

Until recently the organic content of water was generally evaluated using gross analytical determinations such as Total Organic Carbon (TOC), Chemical Oxygen Demand (COD), various extracting methods (Carbon Chloroform Extract, CCE; or Cabon Alcohol Extract, CAE), or Biological Oxygen Demand (BOD). Quantitative determination of individual molecular species present in the microgram per liter range represented a formidable task. The need to understand the specific nature of the array of contaminants present in our water supplies, however, has led to significant progress in the development of methods and instrumentation required for the identification and quantification of such contaminants. Resins, capable of removing specific categories of trace organic compounds, and the development of gas- and liquid-chromatographic techniques have made possible the detection and measurement of many types of both volatile and non-volatile organic compounds from dilute sources.

Measurements of organic carbon in natural waters were reported as early as 1926 by Birge and Juday (24) who investigated the organic matter content of Wisconsin lakes. They found concentrations of 3-13 mg/L organic carbon and 0.14-0.75 mg/L organic nitrogen. In 1934 during a survey of 529 lakes, they reported (25) organic carbon concentrations ranging from 1.2-28.5 mg/L with a mean of 7.7 mg/L. These determinations were based on total solids, and may therefore have had positive errors, attributable to carbonate decomposition.

A summary of the extent of organic impurities in natural waters is presented in Table C-1. One particularly noteworthy study is the Environmental Protection Agency's National Organics Reconnaissance Survey (NORS) which was undertaken in response to the Safe Drinking Water Act of 1974 (40). This act directed EPA to, "conduct a comprehensive study of public water

TABLE C-1. ORGANIC IMPURITIES IN SURFACE WATERS

Water Resource	Constituent	Concentration	Place	Year	Reference
Lake water	organic carbon organic nitrogen	3-13 mg/L 0.14-0.75 mg/L	-	1926	Birge and Juday (24)
529 lakes	organic carbon	1.2-28.5 mg/L (mean = 7.7 mg/L)	-	1934	Birge and Juday (25)
Tap water	organic compounds eluted from carbon filter with diethyl ether	113 μg/L organic-C	Cincinnati	1951	Braus, H. *et al*. (26)
Lake Huron	average organic carbon	3.1 mg/L	-	1963	Van Hall *et al*. (27)
municipal water (unidentified)		1.6 mg/L			
river water (unidentified)		11.8 mg/L			
Municipal waters	average organic carbon	0.9 mg/L 3.2 mg/L	12 cities in AZ, CA, NM, and OR	1968	Nelson and Lysyj (28)
Surface waters	carcinogenic substances	recovered	-	1969	Borneff (29)
				1963	Borneff *et al*. (30)
				1965	Takemura *et al*. (31)
Potable water	chlorinated hydrocarbons	-	lower Mississippi	1970	Sweet (32)

(continued)

TABLE C-1 (continued)

Water Resource	Constituent	Concentration	Place	Year	Reference
Water supplies	carbon chloroform extractable organics	many exceeded Public Health Service's recommended limit (= 200 μg/L)		1970	U.S. Public Health Service (33)
Potable water	chlorinated hydrocarbons		lower Mississippi	1971	Friloux (34)
Surface waters	carbon chloroform extract carbon alcohol extract	0.02-0.57 mg/L (weighted mean = 0.08 mg/L) 0.03-4.6 mg/L (weighted mean = 0.21 mg/L)	129 stations throughout United States	1957 1972	Committee Report (41)
Raw and finished water supplies	46 organic chemicals	trace amounts	lower Mississippi	1972	E.P.A. (35)
Surface water	di-n-butyl phthalate dibutoxyethoxymethane di-2-ethylhexyladipate di-octylphthalate di-isodecylphthalate	1-30 ppb	Monatiquot River, MA	1972	Hites and Biemann (36, 37)
Surface water	biphenyl, trichlorobenzene butylbenzoate	0.1-0.5 ppb	Merrimack River, MA	1973	Malcolm and Leenheer (38)
Surface water	dissolved organic carbon	0.1-15 mg/L (mean = 1.2 mg/L)	100 sites in 21 U.S. cities	1973	Malcolm and Leenheer (38)
Surface water	dissolved organic carbon	< 1 mg/L	Wilmington, N.C.	1973	Malcolm and Leenheer (38)

(continued)

TABLE C-1 (continued)

Water Resources	Constituent	Concentration	Place	Year	Reference
Surface water	$CHCl_3$	6.0-54.0 µg/L	stored surface water	1974	Rook (9)
	$CHCl_2Br$	4.3-20.0 µg/L			
	$CHClBr_2$	1.7-13.3 µg/L			
	$CHBr_3$	1.1-10.0 µg/L			
Potable water	chloroform	37.3-152 µg/L	tap water from various municipal supplies	1974	Bellar, Lichtenberg, and Kroner (10)
	bromodichloromethane	2.9-20.8 µg/L			
	dibromochloromethane	0.1-2.0 µg/L			
Natural waters	16 organic compounds	identified		1975	Pitt, Jolley, and Scott (39)
Raw and finished water supplies	chloroform	trace-0.9 µg/L in 62% of the sampled raw water; 0.1-311 µg/L in all of the sampled finished water.	National Organics Reconnaisance Survey of 80 water supplies	1975	E.P.A. (21)
	non volatile organic carbon	< 0.05-12.7 mg/L (median = 1.5 mg/L			Symons et al. (40)
	85 organic compounds	identified			
Surface water	total organic carbon	7-45 mg/L	Minnesota river basins	1975	Maier et al (23)
Surface water	total organic carbon	5 mg/L	Lake Superior	1975	Maier et al (23)
Drinking water	187 compounds	identified	U.S.	1975	Mullaney (42)

(continued)

TABLE C-1 (continued)

Water Resource	Constituent	Concentration	Place	Year	Reference
Surface waters	33 and 22 trace organic compounds	identified	2 Philadelphia water supplies	1976	Suffet et al. (43)
Surface waters	140 trace organics chloroform	found present in over 80% of the samples	around industrial centers in the U.S.	1976	Chian et al. (44)
Natural waters Drinking waters	16 organics 8 organics	μg/L levels μg/L levels		1976	Pitt, Jolley, and Katz (45)
Drinking waters	72 organics	identified	5 supplies in U.S.	1976	Coleman et al. (46)
Drinking water	117 peaks in carbon chloroform extract	found	13 U.S. cities	1976	Keith et al. (47)
Drinking waters	27 classes of 360 organic solutes	identified at concentrations < 1 ppb	England	1976	Newell (48)
Surface waters	160 acid extractable compounds 89 base extractable compounds 81 purgeable organic compounds	found	204 sites in S.W. U.S. near heavily industrialized areas	1977	E.P.A. (49)
Drinking water	700 trace chemicals	reported from previous findings	U.S. cities	1978	E.P.A. (4)

supplies and drinking water sources, to determine the nature, extent, sources, and means of control of contamination by chemical or other substances suspected of being carcinogens (50)." The survey confirmed that the problem of organic compounds in drinking water was widespread and that trihalomethanes were present in µg/L quantities in most finished drinking waters as a result of chlorination (51). The most recent comprehensive data on the presence of organic chemicals in drinking water are found in the National Organics Monitoring Survey (NOMS) of 1976 and 1977 (4,52). The NOMS was intended to provide a more comprehensive survey of synthetic organic contamination in finished drinking water by monitoring 21 specific organic compounds, and four general parameters of organic content in 113 community water supplies. The compounds were selected on the basis of possible occurrence, available toxicological data, and the existence of analytical methodology for their identification and quantification. The general parameters monitored included: 1) total nonpurgeable organic carbon; 2) carbon chloroform extract; 3) ultraviolet absorbance; and 4) emission fluorescence. Initial results (52) indicated that the occurrences and concentrations of trihalomethanes in finished water were greater than for any of the other selected compounds studied in the survey. Total trihalomethane concentrations ranged from 0.02 to 550 µg/L. Many of the selected contaminants were frequently found at low concentrations in many of the cities. Supplies located on rivers downstream from large industries were more susceptible to raw-water contamination. General parameters did not correlate with the occurrences of specific organic compounds.

Although large strides have been made towards quantifying the vast array of organic contaminants in water supplies, the identity of only 40% by weight of the non-humic material present has been established (21,11). Table C-2 shows the percentage of organic compounds identified in different categories of materials present in natural waters. About 90% of the volatile organic compounds has been identified, as compared to only 30-60% of the non-humic non-volatile constituents (53,11). Included in the larger fraction of unidentified compounds is the category of compounds comprising the topic of this report: nitrogenous organic materials. The non-volatile property of most of these substances has made their identification and analysis by gas chromatography difficult without the prior formation of volatile derivatives. Recent advances in the field of high performance liquid chromatography (HPLC), however, have made the detection of non-volatile nitrogenous compounds increasingly feasible.

NITROGENOUS ORGANIC COMPOUNDS

Although previous attention has been focussed on the environmental hazards of non-polar chlorinated organic contaminants, nitrogenous organic compounds are also environmentally significant. Many naturally occurring nitrogenous organic compounds react readily with aqueous chlorine exerting significant chlorine demand (54,55). Guter, Cooper and Sorber (56) hypothesized that N-chloro compounds were formed during the chlorination of polluted waters and that some of these react more readily with supposedly selective reagents for free active chlorine than do the ammonia chloramines. Several types of N-chloro organic compounds have been identified that yield

TABLE C-2. COMPOSITION OF MATERIALS IDENTIFIED IN NATURAL WATERS

	Classification		
	Volatile	Non Volatile	
		Humic	Non Humic
Approximate % composition in natural waters	10	75 [a]	15
% of organic compounds identified in classification	90	The specific structures of the humic substances have not been fully established	5-10 (of total non volatile compounds) 33-67 (of non-volatile non humic compounds)
% of total compounds identified in natural waters	9	-	4.5-9
% of non humic compounds identified in natural waters	36	-	18-36

[a] Humic substances comprise the major portion of the organic material in natural waters. Percent composition of total organic material in different waters will vary.

interference or false positive tests in determining free chlorine (57). Because these combined forms are generally much less germicidal than free aqueous chlorine, falsely positive tests for free chlorine may indicate a nonexistent bactericidal or virucidal behavior. Reliable measurements of the free chlorine present in water supplies or wastewater containing nitrogen can, therefore, not be made unless the types of organic nitrogen compounds present are known.

Reactions of a broad range of nitrogenous organic substances representative of materials thought likely to occur as part of the organic nitrogen of water sources have been studied (54, 234) and some (m-aminophenol, uracil, tryptophan, pyrrole, chlorophyll, alanine, proline, ℓ-hydroxyproline, and indole) have been found to be potential precursors of trihalomethanes in the chlorination of water supplies. Purine and pyrimidine bases such as caffeine, cytosine, and uracil (commonly found in wastewaters) have been shown to produce complex stable mutagenic chlorinated products upon chlorination (58-65) suggesting that N-chloro compounds may be significant intermediates for the compounds causing mutagenic activity in finished waters. 5-chlorouracil was also shown to effect the hatchability of carp eggs (66) and to cause abnormalities in the larval fish produced (67). In addition certain types of nitrogenous compounds which may be present in polluted drinking water sources are themselves potential health hazards or direct precursors. Included among these compounds are secondary amines capable of forming carcinogenic nitrosamines (68), nitroaromatic compounds, and some hetero-cyclic materials. Some trace nitrogen containing polycyclic compounds are known carcinogens (69). Brown *et al.* (70) found the formation of local sarcomas in rats following subcutaneous injection of unspecified oxides of purines. Nitro and amino derivatives of pyrrole may also be carcinogenic (71). Tryptophan and two related compounds, indole, and indolacetic acid have been shown to enhance the incidence of bladder cancer in certain closely defined situations (72-76).

Organic nitrogen enters the environment via the pathways previously described for organic substances in general. Table C-3 summarizes the sources and properties of selected nitrogenous organic compounds. The chemical structures of some of these substances are shown in Appendix B, Figure B-10. Several studies have begun to characterize the organic nitrogen compounds present in domestic sewage (39, 81-84) and urine (85). Organic nitrogen concentrations have been reported to be between 0.064 mg/L - 0.24 mg/L in several English lakes (86) and from trace amounts to 0.28 mg/L in streams of southern New Jersey (87). Ram (87) reported that the organic nitrogen fraction ranged from traces to 15.6% of the total Kjeldahl nitrogen in streams of southern New Jersey. Some attempts have been made to distinguish and determine amino acids in natural waters (88-90). The quantities found, some μg/L of nitrogen *in toto* seem small compared with the total organic nitrogen expected. Briggs (91) estimated that free amino acids were present at concentrations of approximately 10^{-9} g/L in water while Semenov and his coworkers (92) reported levels of free amino acids in surface waters of the Soviet Union ranging from 2-25 μg/L. Peptide organic nitrogen has been reported to comprise between 15-43% of the total organic nitrogen in surface waters (86,93) with concentrations in the range of 10^{-4} mg/L (91). A summary of the organic nitrogen contents found in natural waters is shown in Table C-4.

TABLE C-3. SOURCES AND PROPERTIES OF SELECTED NITROGENOUS ORGANIC COMPOUNDS (77-80, 54, 41)

Compound	Natural Sources	Man Made Sources	Properties
Adenine	a purine; nucleic acid unit. widespread throughout animal and plant tissues; constituent of nucleic acids and coenzymes		MW [a] = 135.14 LD_{50} orally in rats 745 mg/kg[b] λ_{max} = 256.4 (H_2O)[c]
Alanine	an amino acid. in soil: silty loam: 6-160 µg/kg clay soils: 30-400 µg/kg	excreted in urine: 0.55 mg/kg body wt per day; in domestic sewage: 5 µg/L	MW = 89.09 λ_{max} = no maxima at pH 8 potential trichloromethane precursor
m-Aminophenol	derivative of phenol, coal tar		MW = 109.12 LD_{50} = 0.144 g/kg in white mice LD_{50} = 1 g/kg in rats
Aspartic acid	an amino acid. occurs in animals and plants	exreted in urine: 0.37-3.7 mg/kg body weight per day	MW = 133.1
Barbituric acid	pyrimidine derivative		MW = 128.09 λ_{max} = 257 (neutral)

(continued)

TABLE C-3. (continued)

Compound	Natural Sources	Man Made Sources	Properties
Caffeine	a purine; occurs in tea, coffee, and maté leaves, guarana paste and cola nuts	undisinfected sewage effluent: <1mg/L; Raw sewage after activated sludge treatment	MW = 194.19 LD_{50} of monohydrate orally in rats: 200 mg/kg body weight
Chlorophyll	green pigment of plants		MW = 907.46 potential trichloromethane precursor
5-Chlorouracil		in chlorinated sewage effluent: .0043 mg/L	mutagenic
Creatine	present in muscular tissue of many vertebrates	not found in normal human urine	MW = 131.14 λ_{max} = 215 (BuOH/EtOH/H_2O pH 8)
Creatinine	occurs in all soils and in grain seeds and other vegetable matter	normal constituent of urine: 25 mg/kg bodyweight per day	MW = 113.12 λ_{max} = 210 (BuOH/EtOH/H_2O pH 8)
Iso cyanuric acid		formed on heating urea; base for swimming pool chlorination	MW = 129.08 very toxic to certain types of barley and radishes λ_{max} = 182 (mono ion) 198 (di ion) 228 (tri ion)

(continued)

Table C-3. (continued)

Compound	Natural Source	Man Made Source	Properties
Cytosine	a pyrimidine; nucleic acid unit, widely distributed in nature, constituent of yeast and of wheat embryo		MW = 75.07 λ_{max} = 270 (water)
Glutamic acid	an amino acid	excreted by man in urine 1.8-11.5 mg/kg body wt per day	MW = 147.13
Glycine	an amino acid; gelatin and silk fibroin are best sources normal constituent of proteins	excreted by man in urine 2.3-18.0 mg/kg body wt per day	MW = 75.07 λ_{max} = 630 (water, copper chelate)
Glycylglycine	simplest of all peptides		MW = 132.12; λ_{max} = no maximum at pH 7
Guanine	a purine; nucleic acid unit occurs in animal and vegetable tissues, in excreta; fish scales		MW = 151.13 λ_{max} = 248 (cation)
ℓ-Histidine	an amino acid	excreted by man: in urine: 0.98-6.6 mg/kg body wt/day in feces: 1.4-2.1 mg/kg body wt/day in sweat: 6-10 mg/100 ml	MW = 155.16 λ_{max} = 217.5 (pH=7)

(continued)

TABLE C-3. (continued)

Compound	Natural Source	Man Made Sources	Properties
ℓ-Hydroxyproline	an amino acid	excreted by man in urine: 0.02 mg/kg body weight per day	MW = 131.13 potential trichloromethane precursor
Indole	constituent of coal tar natural pigments	excreted in human feces, contents of domestic sewage: 0.25 μg/L	MW = 117.14 λ_{max} = 278 (H_2O) potential trichloromethane precursor
Leucine	an amino acid	excreted by man: in urine: 0.2-0.52 mg/kg body wt/day in feces: 4.3-6.9 mg/kg body wt/day in sweat: 1.2-4.2 mg/100 ml	MW = 131.7 no maximum U.V. absorption at pH 8
ℓ-Proline	an amino acid	excreted in man in urine: 0.3-0.9 mg/kg body wt/day	MW = 115.13 λ_{max} = 200 (MeOH) potential trichloromethane precursor
Pyridine		constituent of coal tar	MW = 79.1 faint odor at .0037 mg/L; acute oral LD_{50} in rat = 0.8-1.6 g/kg; transient symptoms in man: 125 ppm, 4 hrs/day, for 1-2 weeks; λ_{max} = 250 (H_2O)

(continued)

TABLE C-3. (continued)

Compound	Natural Source	Man Made Source	Properties
Pyrimidine	basic heterocyclic ring		MW = 80.09 λ_{max} = 200 (MeOH) potential trichloromethane precursor
Pyrrole	building block for chlorophyll hemoglobin, hemocyanin, etc.	constituent of coal tar and bone oil	MW = 67.09; minimum lethal dose: in mice = 60.5 g/kg; potential trihalomethane precursor; λ_{max} = 205 (H_2O)
Sarcosine	found in star fishes and sea urchins		MW = 89.09
dℓ-Serine	an amino acid	excreted by man in urine 0.35-1.4 mg/kg body wt/day	MW = 105.09
Succinimide			MW = 99.09
Taurine	present in bile, lungs and flesh of oxen, shark blood muscles, oysters		MW = 125.14
Thymine	pyrimidine derivative, isolated from thymus; nucleic acid unit		MW = 126.11 λ_{max} = 266
Tryptophan	an amino acid	excreted in man in urine: 0.23-1.3 mg/kg body wt/day also found in human saliva & blood	MW = 204.22; λ_{max} = 278 (pH 7); potential trihalomethane precursor

(continued)

TABLE C-3. (continued)

Compound	Natural Sources	Man Made Sources	Properties
Tyrosine	widely distributed amino acid in soil: silty loam: 0-65 μg/kg soil clay soils: 10-60 μg/kg soil	excreted in man in urine: 0.35-1.45 mg/kg body weight per day	MW = 181.19 λ_{max} = 274 (pH 7)
Urea	product of nitrogen metabolism in mammals	contents of domestic sewages: 2-6 mg/L; domestic sewage effluent: .020 mg/L	MW = 60.06 λ_{max} = 260 (pH 9.5)
Uracil	pyrimidine derivative hydrolysis product of nucleic acids	in sewage effluent: 0.013 mg/L	MW = 112.09 λ_{max} = 203, 258 (pH 7) potential trihalomethane precursor
Uric acid	chief end product of the nitrogenous metabolism of birds and scaly reptiles, found in their excrement; present in urine of all carnivorous animals	contents of domestic sewages: 0.2-1.0 mg/L	MW = 168.11

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[a] MW = molecular weight

[b] LD_{50} calculated lethal dose expected to kill 50% of an experimental animal population

[c] ( ): parentheses indicate solvent or conditions used in absorbance measurement

TABLE C-4. CONCENTRATION OF TYPES OF ORGANIC NITROGEN IN SURFACE WATER

Water Resource	Constituent	Concentration	Place	Reference
Natural Waters	amino acids	1 μg/L nitrogen *in toto*		Gardner & Lee (88) Peake, Baker & Hodgson (89) Georgiadis & Coffey (90)
Lake Water	peptide-N	0.056-0.436 mg/L	Lake Mendota Lake Michigan	Domogalla, *et al*. (93)
Lake Water	total organic nitrogen	0.064-0.24 mg/L	England	Fogg & Westlake (86)
River Water	total organic nitrogen	trace-0.28 mg/L	Southern N.J.	Ram (87)
Lake, estuary, and ocean waters	dissolved free amino acids	found		Grob (94)
Lake Water	particulate organic nitrogen	approximately 15% of the particulate organic carbon	N.E. Wisconsin	Croll (224)

(continued)

TABLE C-4. (continued)

Water Resource	Constituent	Concentration	Place	Reference
Lake Water	free amino acids; peptides	$\sim 10^{-9}$ g/L $\sim 10^{-7}$ g/L	North Island, New Zealand	Briggs (91)
Tap Water	free amino acids	none found	London, England	Sidle (95)
Surface Water	free amino acids	2-25 µg/L as N	Soviet Union	Semenov (92)
	proteins	20-340 µg/L as N		
	volatile amines	6-100 µg/L as N		
Surface Water	peptides	226-250 µg/L		Newell (96)
	common amino acids (serine and glycine)	36-61 µg/L		
River Water	pyrrole		Rhine River	Holluta (98)
Hydrolyzed tap water	isoleucine + leucine	6.03 µg/L	London, England	Sidle (95)
	valine	15.23 µg/L		
	glycine	6.45 µg/L		
	alanine	3.83 µg/L		
	glutamic acid	5.00 µg/L		
	aspartic acid	3.06 µg/L		
River Water	aromatic amines		Rhine River	Holluta & Talsky (97)
	aliphatic amines			
	proline			
	tryptophan			
	indole			
	skatole			
	hydroxybenzamide			

(continued)

TABLE C-4. (continued)

Water Resource	Constituent	Concentration		Place	Reference
Lake Water hydrolysates	glycine, α-alanine, aspartic acid, glutamic acid, methionine sulphoxide, arginine, glycine, hydroxyproline, isoleucine, leucine, lycine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine and tyrosine			North Island, New Zealand	Briggs (91)
Receiving Water below an industrial plant	N-containing heterocycles and other nitrogen containing compounds				Jungclaus et al. (99)
	Compound:	Drinking Water	River Water	Ground Water	
Drinking water, river water, and ground water	aspartic acid	.67 µg/L	2.66 µg/L	1.33 µg/L	Kasiske et al. (231)
	serine	1.05 µg/L	4.20 µg/L	1.58 µg/L	
	glutamic acid	.74 µg/L	4.41 µg/L	.74 µg/L	
	glycine	.75 µg/L	4.50 µg/L	1.50 µg/L	
	alanine	.45 µg/L	.89 µg/L	.45 µg/L	
	leucine	.33 µg/L	.33 µg/L	.33 µg/L	
	phenylalanine	.83 µg/L	.83 µg/L	-	

ALGAL PRODUCTION OF N-ORGANIC EXTRACELLULAR METABOLITES

Blue-green algae have received considerable attention as major nuisance algae because they proliferate in waters where conditions are unfavorable for most other types. They are usually found in neutral to alkaline waters, are able to tolerate a relatively wide temperature range, and are often associated with taste and odor problems (100, 101). Although most algae are known to liberate organic compounds in general into the surrounding aquatic environment during growth (102), blue green algae are known also to liberate comparatively large amounts of organic nitrogen (86, 103, 104). Up to 50% of the carbon fixed by a population can be released (105). The amount of release per cell is known to be inversely related to population density (102,106,107), and is favored by high pH (108) and inorganic nutrient deficiency (105,109). (High oxygen)-(low carbon dioxide) concentrations and low light intensities typical of conditions in natural waters also favor release (104). Greatest liberation in laboratory grown cultures of blue-green algae occurs during the exponential phase of growth (104-106,110) although Thompson (104) observed a second per cell increase in liberated total organic carbon in late growth. Thompson (104) also reported a linear increase in secreted TOC with time and a linear increase of TOC with time except for an initial peak production at about ten days for laboratory grown cultures of *Oscilaatoria tenuis* and *Anabaena flow aquae* respectively after a two-day lag-period in both. Various authors have reported release of leucine, serine, alanine, aspartic acid, valine (103,111), alanine, aspartic acid, arginine, leucine, proline, valine (112), glycine (103), and other amino acids, polypeptides and proteins (102, 103,113,114).

These reports suggest that isolation and identification of N-organic compounds in water supplies can be facilitated by sampling during a blue-green algal bloom.

UROCHROMES AND HUMIC SUBSTANCES

Urochromes are yellow pigments discharged in human urine as the metabolic product of porphyrins (115). They are found in urine (116-119), sewage (10-243 mg urochrome per liter), water from drainage canals (3-14 mg urochrome per liter) and treated surface waters (0.6-1.5 mg urochrome per liter) (120). Their presence in water sources is a clear indication of fecal contamination and is therefore of extreme concern from the standpoint of public health (115,121). In addition to the indirect hazards arising from their association with enteric bacteria (121), their presence in water has been related to the occurrence of endemic endocrine goiter in humans (122-124).

Elementary analyses of urochromes oxy-A and B have shown them to be comprised of about 2.44% and 2.61% nitrogen respectively (122,125). Since they have some of the nitrogenous structure of pyrrole and porphyrin they are potential haloform precursors. They are therefore, considered in this report.

The enrichment and isolation of urochromes from water samples according to Hettche (122,126,127) is carried out by adsorption onto aluminum oxide or entrainment precipitation with alum and ammonia. After elution with formic acid and dilution to a known volume they are determined by absorptiometry at 380 nm. Both humic acids and lignin derivatives interfere with quantitative evaluation of urochromes (121,115). Humic substances are high-molecular-weight compounds comprising an unresolved range of complex polymeric aromatic organic acids. They result from the aqueous extraction of the soluble fraction of wood tissues, dissolution of decomposition products of decaying wood and/or leaching of soluble soil components (26,94). Knorr *et al.* (128) could not distinguish humic acids from urochromes A or B infrared or ultraviolet spectroscopy, electrophoresis, fluorescence, surface tension, or nitrogen content. Hettche (122) proposed distinguishing between urochromes and humic acids by computing the difference in the logarithms of the absorbance values at 380 and 530 nm for urochromes and humic acid respectively, and multiplying by 1000. A value equal to 0.9 for pure urochrome and 0.537 for humic acids, was suggested (115). Sattlemacher and Furstenau (129) however, observed that no specific extinction curve could be plotted for humic substances in the visible region. They concluded that the method of Hettche for the determination of urochromes was inexact. They developed a separation scheme utilizing paper chromatography after esterification with diazooctadecane and extraction with chloroform.

Humic substances are also considered in this report because they form the major part of the organic material in naturally high-colored waters (26). They are derived from vegetative material and have certain similar characteristics. Humification takes place under a wide range of environmental conditions. Being resistent to microbial and chemical decomposition, humus tends to accumulate even under aerobic conditions. Unfortunately, the chemical structures of aquatic humic substances are not known with any certainty (233). Oden (130) classified all humic substances into three groups according to their solubility in strong acid and alcohol:

TABLE C-5. CLASSIFICATION OF HUMIC SUBSTANCES, AFTER ODEN (130)

Classification	Strong Acid	Alcohol
fulvic acid	soluble	-
hymatomelanic acids	insoluble	soluble
humic acids	insoluble	insoluble

Croll (224) reported the elemental analysis on an ash free basis for the humic plus hymatomelanic acids and for the fulvic acids to be: Carbon 51.52%, Hudrogen 4.59%, Nitrogen 2.84% and Carbon 55.61%, Hydrogen 5.91%, Nitrogen 2.13%, respectively.

Because humic substances usually comprise a large percentage of the organic content of natural waters and because they contribute minimally to the total organic nitrogen content (224) it was hypothesized that their removal from water samples might facilitate the isolation of individual nitrogenous compounds. Stuermer and Harvey (131) commented on the hydrophobic characteristics of humic substances, implying the use of selective macroreticular resins for their removal. Since the bulk of organic nitrogen is associated with hydrophilic substances (131,38) it appeared analytically feasible to remove the potentially interfering humic materials from water samples with resins which selectively adsorb hydrophobic compounds. Theoretically, a prefiltration through such a resin bed would not significantly effect the hydrophilic nitrogenous compounds. Generally speaking, macroreticular resins of low and intermediate polarity adsorb hydrophobic solutes, while they do not adsorb hydrophilic solutes (38).

Several studies have been conducted on the adsorption of humic substances onto XAD macroreticular resins (Rohm and Haas Company, Philadelphia, PA). Although Oulman (133) reported that XAD-4 had poor capacity for humic substances, Chen (134), Blunk (135), Stuermer and Harvey (131), and Weber and Wilson (136) observed strong adsorption of humus onto macroreticular resins of varying polarity. Cheng (134) found that adsorption of humic acid by XAD resins was generally favored at neutral conditions (pH 6-7). Blunk (135) adsorbed 97-99% of humic material from untreated river water as assayed by absorbance at 450 nm onto XAD-7. Weber and Wilson (136) adsorbed fulvic acid onto XAD-2 at pH 1 and Stuermer and Harvey (131) reported adsorption efficiencies of greater than 90% for humic substances in seawater acidified to pH 2 onto XAD-2. Junk *et al.* (132) observed an increase in the adsorption of organic compounds onto XAD by adding 5 ml of hydrochloric acid per liter (equal to .056 M or pH of about 1.3) of standard water sample tested. Oliver (233), and Christman (234) acidified water samples to pH 2.0 and pH 2.2, respectively to adsorb humic material in these samples onto XAD resins.

Cheng (134) found that humic substances at pH 5 were more strongly adsorbed onto XAD 1, 2, 4, 7, and 12 than onto XAD-8. Leenheer and Huffman (38) reported that XAD-8 was more effective in adsorbing fulvic acid than were XAD-2 and 4. XAD-8, however, exhibits less irreversible bonding than do XAD-2 and XAD-4 (Leenheer, J.A., personal communication). Its use in the pretreatment filtration step of natural waters is therefore favored.

MACRORETICULAR RESINS

Natural and polluted waters contain a numerous variety of natural and synthetic compounds that seriously interfere with the isolation, identification and determination of individual nitrogenous organic compounds. Rohm and Haas macroreticular resins are known to adsorb selectively a broad range of hydrophobic organic compounds (Table C-6). These undesired or interfering carbonaceous organic materials may be removed by filtration through appropriate XAD resins prior to concentration and chromatographic separation without significant reduction in most nitrogenous constituents, with the exception of heterocyclic aromatic substances.

XAD macroreticular resins are hard insoluble beads, 20-50 mesh, varying from white to light brown in color. XAD-2 and XAD-4 have nonpolar surfaces, XAD-7 and XAD-8 have intermediate surfaces and XAD-12 is highly polar (38).

TABLE C-6. ORGANIC COMPOUNDS ADSORBED ONTO XAD AND TENAX MACRORETICULAR RESINS

Resin	Compounds adsorbed	% recovered[a]	Reference
XAD 2	polycyclic aromatics, n-alkanes, phthalates, halogen compounds, phenols fatty acids, fatty acid methyl esters, steroids	36 - 100 average: 83.27 %	Shinohara et al. (137)
XAD 2	pesticides in natural waters		Junk et al (138)
XAD 2	coprostanol (a characteristic sterol found in the feces of man and higher animals)	97% at flow rate = 3 ml/min	Wun et al. (139)
XAD 2	organic contaminants		Burnham et al. (140)
XAD 2	alcohols, aldehydes, acids, aromatic halides, alkylbenzenes, phenols, esters, ethers, ketones, polynuclear aromatics, herbicides, pesticides and various compounds containing halogens, nitrogen or sulfur	average recovery rate of 110 individual organic solutes was 78% with a standard deviation of 6.3%	Junk et al. (132)
XAD 2	Nitrogen compounds:		Junk et al. (132)
	Hexadecylamine	94	
	Nitrobenzene	91	
	Indole	89	
	O-Nitrotolutene	80	
	N-Methylaniline	84	
	Benzothiazole	100	
	Quinoline	84	
	Isoquinoline	83	

(continued)

TABLE C-6. (continued)

Resin	Compounds adsorbed	% recovered	Reference
	Benzonitrile	98	
	Benzoxazole	92	
XAD 2	visible color of 6 textile dyes	all visible color removed	Webb (141)
XAD 2	alcohols	94	Junk et al. (142)
	aldehydes and ketones	95	
	esters	93	
	acids	101	
	phenols	89	
	ethers	90	
	halogen compounds	87	
	polynucleic aromatics	89	
	alkylbenzenes	90	
	nitrogen and sulfur compounds	89	
	pesticides and herbicides	90	
	weighted average	91	
XAD 2	alcohols	100	Junk et al. (143)
	aldehydes and ketones	74	
	alkanes	5	
	amines	14	
	aromatics	68	
	benzothizoles	67	
	esters and ethers	74	
	halogenated compounds	57	
	PCB's	78	
	phenols	46	
	weighted average	59	

(continued)

TABLE C-6. (continued)

Resin	Compounds adsorbed	% recovered	Reference
XAD 8	14 compounds previously identified in industrial effluents	average: 63.31	Webb (141)
XAD 2, 4, 7 and 8 and mixtures	13 organic pollutants	an equal weight mixture of XAD 4 and XAD-8 was most efficient	Van Rossum and Webb (144)
XAD General	phenols, alkyl sulfonic acids, dyes, steroids, vitamin B-12, fulvic acid	many non-ionic organic compounds extracted from dilute aqueous solution with approximately 100% efficiency	Burnham *et al*. (145)
XAD-2 XAD4/8 Tenax GC	20 compounds representing aliphatic hydrocarbons, aliphatic and aromatic halogenated hydrocarbons, phthalates, polynuclear aromatic hydrocarbons	All of these resins gave comparable results of 64% average recovery for equal volumes and equal weights of resin. Tenax averaged 80%; XAD-2, 64%; and XAD 4/8, 69% for equal surface areas of resin	Webb (146)

[a] after elution from resin with appropriate solvent

XAD-2 and XAD-4 are styrene-divinyl benzene copolymers while XAD-8 is an acrylate ester. XAD 2, 4 and 8 have average surface areas of 330 m^2/g, 750 m^2g and 140 m^2/g respectively and pore sizes of 90 angstroms, 50 angstroms and 250 angstroms, respectively (141). Surface adsorption is the principal contributing factor to retention on the XAD resins (147,141). No ion-exchange mechanism is involved (141). Leenheer and Huffman (38) reported the absorptive capacity of XAD to vary from 5-20 mg organic carbon per gram of resin for different hydrophobic organic compounds.

Liquid-liquid extraction and carbon adsorption were also considered as alternative methods for removing interfering hydrophobic materials from water samples. Each of these methods, however, has several significant draw-backs in comparison with the XAD resins (Table C-7), and were therefore not employed.

In addition to the XAD series other macroreticular resins have been developed for selective adsorption of organic compounds from water. Ambersorb XE 340 (Rohm and Haas, Philadelphia, PA) resin was recently designed to remove nonpolar hydrophobic organic compounds or halogenated organic molecules from the aqueous or gaseous phase (148). It is comprised of hard, nondusting, black spheres whose chemical composition is intermediate between that of activated carbon and polymeric substances. It has a surface area (N_2, BET method) of 400 m^2/g and a pore size distribution ranging from 6-300 angstroms (148). XE 340 has a high capacity for removal of low molecular weight chlorinated organic compounds such as chloroform (148).

Tenax GC (Applied Sciences Laboratory Inc., State College, PA) is a porous polymer based on 2,6-diphenyl-p-phenylene oxide. It is designed as a gas-chromatographic support for the separation of high-boiling polar compounds such as alcohols, polyehtylene glycol compounds, diols, phenols, mono- and diamines, ethanolamines, amides, aldehydes, and ketones (150). It has been used to concentrate organic compounds from air (151) and has been suggested as a useful direct accumulator for organic compounds from water in a manner similar to XAD resins (146). Novotmy (152) proposed that Tenax be used as an adsorbant for a wide range of both polar and nonpolar compounds. Webb (146) found significantly better recoveries of phenol (72%), camphor (83%), and alphaterpineol (87%) on a 13 cm Tenax column in comparison with a comparable XAD column. Twenty organic compounds were recovered with 80% efficiency on the Tenax resin.

ISOLATION OF TRACE ORGANIC COMPOUNDS FROM DILUTE AQUEOUS SOLUTION

Determination of trace nitrogenous organic compounds, present at only nanogram levels, requires several analytical procedures: 1) selective adsorption of extraneous interfering organic compounds with macroreticular resins; 2) concentration of the water sample to achieve detectable elevels of dissolved solutes; 3) separation and detection of constituent compounds using high pressure liquid chromatography; and 4) identification of resolved constituents. Selective adsorption of the hydrophobic portion of the dissolved organic carbon in natural waters was considered in the previous section.

TABLE C-7. COMPARISONS WITH XAD RESINS

Method	Comments	Reference
Carbon Adsorption	difficult to extract large volumes of water containing small amounts of organic pollutants	Van Rossum and Webb (144)
	unacceptable background contamination values	Van Rossum and Webb (144)
	incomplete recovery of compounds	Webb (141)
	non-specific adsorption of compounds	Webb (141)
	irreversible adsorption of solutes	Burnham et al. (145) Malcolm and Leenheer (38)
	lack of adsorption/desorption control	Sproul and Ryckman (149)
	bacterial and oxidizing attack on the adsorbed organic compounds	Sproul and Ryckman (149) Burnham et al. (145)
	meticulous purification required	Burnham et al. (145)
Solvent Extraction	distribution coefficient for the contaminants between water and an extracting solvent may be unfavorable	Burnham et al. (145) Van Rossum and Webb (144)
	labor requirement excessive	Webb (141) ; Van Rossum and Webb (144)
	difficult for highly polar organic solutes comprising a major fraction of organic solutes	Malcolm and Leenheer (38)
	should be convenient to extract hydrophobic compounds without extracting hydrophilic substances	

(continued)

TABLE C-7. (continued)

Method	Comments	Reference
Macroreticular Resins	selectively adsorb compounds of different polarity	
	low energy requirements, not involving any phase transition.	
	low temperature and high vapor pressure at which adsorption occurs	
	favorable kinetics	Rohm and Hass publication (148)
	homogenious surface	Malcolm and Leenheer (38)
	high surface area and capacity	
	good stability	
	only one adsorption mechanism operative, allowing solutes to be quantitatively sorbed and desorbed	
	good hydraulic flow characteristics for column operation	

Numerous methods of concentration have been reported in the literature including: liquid-liquid extraction, freeze concentration, adsorptive bubble separation, chromatography, ion exchange, ultra-filtration, adsorption, distillation, evaporation, sublimation, and reverse osmosis (26,53,16). Considerations relative to the choice of concentration method are: 1) the desireability of maintaining the sample at a low temperature to avoid decomposition or reaction of solutes; 2) the necessity of collecting the solids that separate during concentration in order to redissolve coprecipated organic compounds; 3) the need to reduce the volume of sample within a reasonable period of time; and 4) the requirement that the method of concentration not alter or chemically degrade the constituents. Extraction and adsorption do not quantitatively concentrate all compounds of interest (81,154). Low-temperature vacuum-distillation appears to be the most desirable method, since it provides efficient recovery of stable, nonvolatile organic compounds and fulfills the requirements described above. This method cannot, however, be used for concentrating unstable or volatile compounds. Low-temperature vacuum-distillation followed by removal of residual water by lyophilization has been used with great success by many workers (39,53,82,83,153,154).

Recent advances in high-performance liquid chromatography (HPLC) have made the separation and determination of nonvolatile nitrogenous organic compounds analytically feasible. The ability to grade resins selectively into narrow and uniform microparticles, advances in design of high-pressure columns and pumps, and the development of sensitive detection systems have resulted in an increased range of chromatographic capability.

Chormatography can be characterized as a separation method based on the differential migration of solutes through a system of two phases, one of which is mobile. The basis of the chromatographic separation is the distribution (or partition) of the sample components between two phases, which are immiscible. Chromatographic methods are classified either according to the type of mobile and stationary phases utilized or according to the mechanism of retention. A description of many modes of chromatography is presented in Table C-8. Ion-exchange chromatography has been used extensively in the separation of amino acids, and more recently, in the separation of nucleic acid components (155). It was, therefore, closely examined as background for this study. Paired-ion chromatography, previously applied to inorganic compounds, has also been used successfully in the separation of very polar compounds (156). In this method a large organic counter-ion added to the mobile phase forms a reversible ion-pair complex with the ionized sample. This complex behaves as an electrically neutral and non-polar (lipophilic) compound. The extent to which the ionized sample and the counter-ion form an ion-pair complex affects the degree of retention obtained (157). Reversed phase adsorption chromatography has also been shown to be useful (155).

Amino acids commonly found in protein hydrolysates, physiological fluids (90) and standard amino acid mixtures (90,158) have been resolved at the picomole level using HPLC with fluorescence detection. Dr. R. Jolley and Dr. W. Pitt have made significant progress in the separation and identification of trace compounds in urine (159), primary and secondary stages of municipal sewage treatment plants, and natural waters (53,79,82), using a strongly basic anion-exchange resin (Bio Rad Aminex A-27) with (ammonium acetate)

TABLE C-8. MODES OF CHROMATOGRAPHY

	Mobile Phase	
Stationary Phase	Non Polar Liquid	Polar Liquid
polar solid	normal phase liquid-solid chromatography [a]	
non-polar solid		reversed phase liquid-solid chromatography
solid surface coated with a polar liquid	normal phase liquid-liquid chromatography	
solid surface coated with a non-polar liquid		reverse phase liquid-liquid chromatography
ion-exchange resin with either acidic or basic mobile counter-ions		ion exchange chromatography
non-polar solid		paired-ion exchange chromatography
porous packing gel	gel permeation chromatography or exclusion chromatography	

[a] also called adsorption chromatography

(acetic acid) buffer as eluant. A summary of the chromatographic supports used to separate various mixtures of nitrogenous organic compounds is shown in Table C-9.

Different modes of operation and chromatographic columns were evaluated in this study, including: cation-exchange chromatography (Zipax SCX, DuPont Company), anion-exchange chromatography (Aminex A-27, Bio Rad Laboratories), paired-ion chromatography (Zorbax CN, DuPont Company), reversed-phase chromatography (Zorbax C8, DuPont Company), and normal-phase chromatography (Corasil II, Waters Associates). Zipax SCX is a strongly acidic, cation exchanger consisting of a sulfonated fluorocarbon polymer bonded to spherical glass microbeads nominally 30 microns in diameter (190,186). These beads have a surface are of 0.8-1.0 m^2/g. It can be used only with water solutions having pH values ranging from 2-9. Ionic strength has the greatest effect on solute retention.

Zorbax particles are tiny, uniform, silica-sol beads which have been produced by agglutination in a polymerization process. Zorbax C8 is a reversed-phase packing for compounds with moderate to high polarity and can also be used in paired-ion chromatography. Zorbax CN is a polar-bonded phase for both normal and reversed phase chromatography (190,157). Aminex A-27 is a porous, moderately cross-linked, polystyrene based, quarternary ammonium type strong anion exchange resin. Corasil II is a normal-phase column packing consisting of a solid glass bead core with either single or double porous silica layers.

Eluted compounds have been detected by various methods including: refractive index, ultraviolet and fluorescence absorbance, heat of adsorption, electrolytic conductivity, flame ionization, polarography, and dielectric constant monitoring. Because of its relatively high sensitivity to most solutes and its insensitivity to changes in temperature, flow rate, and mobile phase composition, ultraviolet adsorption is the most widely used method with HPLC. When ultraviolet detection is used at very short wavelengths, near 200 nm, it becomes a general non-specific detector, since almost all compounds exhibit very strong adsorption in the far U.V. (192).

Identification of a resolved chromatographic peak may be accomplished by: 1) elution position; 2) U.V. spectrum; 3) fluorescence spectrum; 4) nmr spectrum; 5) internal standard; 6) isotopic labeling; 7) enzymatic peak shift techniques; and 8) derivatization methods. Positive identification usually requires corroborative evidence from several identification methods. The probability of correct identification increases with each additional unit of information that shows correspondence between the unknown and known reference standards. Katz and Pitt (159) have recently developed a new liquid chromatography detector which depends upon the fluorescence measurement of cerium III produced from the reaction of cerium IV with eluted reducible compounds. It was reported to be more sensitive than previous oxidative detectors by more than a factor of 100. Gomez and others (193) have begun to try to develop a sensitive method involving dual-beam Fourier transform infrared spectrosocopy for the on-line identification of organic water pollutants separated by high pressure liquid chromatography.

TABLE C-9. HPLC SEPARATION OF NITROGENOUS ORGANIC COMPOUNDS

Compounds	Column	Mobile phase	Reference
nucleic acid bases	Zorbax CN[a] paired-ion chromatography	propionic acid with heptane sulfonic acid	DuPont Co. (160)
nucleic acid bases	Zipax SCX[a]	0.01 HNO_3	Kirkland (161)
nucleic acid hydrolysis products: purines, pyrimidines, nucleosides, RNA hydrolyzates, nucleotides	XAD-4 support coated with triethylammonium bicarbonate	linear gradient, 0.1-0.4 M triethylammonium bicarbonate	Vematsu and Suhadolnik (162)
nucleosides	Aminex A-28[b]	Na Borate at pH 3-9 with varying molarity	Schneider and Glazko (163)
nucleotides	Spherisorb 10µ[c]	tetra-_n_-butylammonium hydrogen sulfate and 10-camphorsulfonic acid pH 3.9	Hoffman and Liao (164)
nucleosides and bases in serum and plasma	µBondapak C_{18}[d]	KH_2PO_4 pH 5.5 60/40 MeOH/H_2O	Strop _et al_. (165)
nucleosides and their bases	µBondapak C_{18}	.01 F KH_2PO_4 pH 5.5 MeOH-H_2O gradient (80/20)	Hartwick and Brown (166)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile phase	Reference
amino acids with fluorescamine detection	DC-4A[e]	Na citrate buffers	Georgiadis and Coffey (90)
dansyl amino acids	Micropak MCH-10[h] ion pairing technique	buffered MeOH/H_2O 0.01 M $(CH_3)_4$ NCl counter ion source	Ellis and Garcia (174)
dansyl amino acids	Micropak-NH_4-10[h]	dichloromethane-acetic acid (99:1)/acetonitrile-acetic acid (90:10) gradient	Johnson et al. (175)
dansyl amino acids	Particil PAC[i] Poragel PN[4] Vydac polar phase[j]	acetonitrile-water-acetic acid	Hsu and Currie (224)
amino acids	resin coated glass beads Poracil C[d] Corasil II[d]	distilled and deionized water pH = 6	Grushka and Scott (176)
urinary constituents	BioRad A-15[b] BioRad A-27[b]	ammonium acetate-acetic acid buffer pH 4.28 (0.015 - 6 M)	Mrochek et al. (177)
urinary U.V. adsorbing metabolites	Zerolit[k]	acetic acid-ammonium acetate (pH 4.4) varying from .015 - 6 M	Geeraerts et al. (178)
urine amino acids	Aminex A-7[b]	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Hamilton (179)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile phase	Reference
purine nucleotide, nucleoside and base metabolites from biological extracts	μBondapak C_{18}	.05 M ammonium dihydrogen phosphate buffer	Anderson and Murphy (167)
adenosine in the presence of other nucleic acid components	μBondapak C_{18}	.007 F KH_2PO_4 H_2O/MeOH gradient	Hartwick and Brown (168)
amino acids and amino sugars	Aminex A-6	sodium citrate buffers of varying pH	Hadzija and Keglevic (169)
fluorescamine derivatized amino acids	Durrum DC-1A[e]	citrate buffers pH 3.28 and 4.25	Stein *et al*. (170)
O-phthalaldehyde derivatives of amino acids	Aminex A-6	citrate buffers: pH 3.2, 4.25, and 6.4	Roth and Hampai (171)
amino acids as dansyl derivatives with fluorometric detection	LiChrosorb SI60[f] LiChrosorb RP8	benzene-pyridine-acetic acid mixture	Bayer *et al*. (172)
ninhydrin chromagens of amino acids	Technicon[g] chromobeads, type B	citrate buffers pH 2.88 and 5.00	Ellis and Garcia (173)
dansyl amino acids	Zipax R[a]	methyl ethel ketone with light petroleum	Frei and Lawrence (158)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile Phase	Reference
serum and urine components	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Katz *et al*. (180)
human urine, blood serum, cerebrospinal fluid, and amniotic fluid	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Katz *et al*. (180)
U.V. absorbing constituents of human urine	Aminex BRX[b]	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Burtis (181)
acidic urinary constituents	LiChrosorb ODS[f]	increasing acetonitrile concentration in dilute acid solution	Molnar and Horvath (182)
U.V. absorbing constituents of human urine	BioRad AGI-18[b]	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Scott (183)
dansyl polyamine derivatives	MicroPak CH-10[h]	water/acetonitrile gradient	Johnson *et al*. (175)
complex biological mixture	Aminex A-27 Aminex A-6	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Scott *et al*. (155)
aromatic bases	Zipax SCX	water with 0.15 M $NaNO_3$	DuPont Co (184)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile Phase	References
cyanopyridines	Zipax SCX	water with 0.10 $NaNO_3$ and 0.1 N H_3PO_4	Talley (185)
Neuroamines, phenethylamines, B-hydroxyphenethyl, amine and indoleamines	Zipax SCX	ammonium phosphate pH 7	McMurtrey (186)
aza arenes	μBondapak C-18 μ Porasil^{d}	20-80% CH_3CN in water 1% propanol in hexane	Dong and Locke (187)
aromatic amine carcinogens	Zipax SCX	0.1 ammonium acetate buffer	Mefford *et al*. (188)
Caffeine in coffee	Zipax SCX	0.01 M nitric acid	Madison *et al*. (189)
organic constituents in primary and secondary sewage treatment plant effluents	strong cation-exchange column	-	Jolley *et al*. (82)
trace organic compounds in municipal sewage effluent	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Pitt *et al* (39)
organic halogen products in chlorinated municipal sewage effluents	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Jolley *et al*. (84)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile Phase	Reference
trace organic compounds	C_{18} µBondapak	20-100% acetonitrile in water	Hites and Biemann (36, 37)
organic compounds extracted from drinking water by adsorption onto XAD macroreticular resins	µPorasil µBondapak	hexane to chloroform + hexane gradient hexane to ethanol + hexane gradient	Thurston (226)
tryptophan and some of its metabolites in biological fluids	Spherisorb ODS	50% MeOH + 50% paired ion chromatographic solution	Riley et al. (227)
benzamide, benzene-sulfonamide, or 4-methoxybenzamide derivatives or primary and secondary amine compounds	µBondapak C_{18}	mixtures of water and MeOH or acetonitrile	Clark and Wells (228)
dansyl amino acids	µBondapak C_{18} Spherisorb 5ODS[1]	linear gradient formed from acetonitrile and sodium phosphate buffers (neutral pH)	Wilkinson (229)
amino acids and aromatic amino acid derivatives	Amberlite CG-120 Type III[b]	gradient from pH = 3.25 to 4.25 to 7.70 using sodium citrate or Borax	Ohtsuki and Hatano (230)

(continued)

TABLE C-9. (continued)

Compounds	Column	Mobile phase	Reference
amino acids	Durrum DC-6A[c]	citrate buffers (pH 3.2, 3.5, and 4.0)	Kasiske *et al*. (231)
mono-, di-, and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, uracil, thymine and cytosine	Partisil 10-SAX[i]	.007 F KH_2PO_4 and .007 F KCl (pH 4.0) to 0.25 F KH_2PO_4 and 0.50 F KCl (pH 5.0) gradient	McKeag and Brown (232)

[a] DuPont Company, Wilmington, Delaware
[b] Bio Rad Laboratories, Richmond, California
[c] Spectra Physics, Santa Clara, California
[d] Waters Associates, Milford, Massachusetts
[e] Durrum Chemical Company, Palo Alto, California
[f] Merck, A.G., Darmstadk, Germany
[g] Technicon Industrial Systems, Tarrytown, New York
[h] Varian Associates, Palo Alto, California
[i] Whatman Inc., Clifton, New Jersey
[j] Separation Group, Hesperia, California
[k] Permutit, London, Great Britain
[l] Jones Chromatography Ltd., Llanbradach, United Kingdom

Fluorescence spectroscopy has long been a valuable method of analysis because of its enhanced sensitivity and selectivity over conventional absorbance spectroscopy. Luminescence phenomea include fluorescence, phosphorescence, chemiluminescence, triboluminescence, and electroluminescence. Fluorescence and phosphorescence are the emission of long wave light following absorption of short excitation wavelength energy. If the energy emission ceases in about 10^{-8} seconds after the excitation source is removed the phenomenon is called fluorescence. If the energy emission persists for a longer time than this, the term phosphorescence is used (194)

Many organic compounds either display natural fluorescence or can be made fluorescent by derivatization. Classes of naturally fluorescing compounds include: catecholamines, polycyclic aromatics, drugs, vitamins, nucleotides, prophyrins, flavins, purines, pyrimidines, coenzymes, dyes and steroids (195). Table C-10 shows luminescence data for a number of nitrogenous organic compounds. The emissions of the purines, pyrimidines, nucleotides, and nucleic acids themselves are weak, especially at ambient temperatures (196). Most amino acids do not fluoresce to any appreciable extent (194). Tyrosine, tryptophan and phenylalanine however, do have fluorescent properties (197).

The fluorescence adsorption and emission spectra of solutes in solution depend greatly on several parameters including: solvent polarity, pH, presence of non-aqueous acids or bases in organic solvents, anion presence (Cl^-, Br^-, I^-, and NO_3^-), and solute concentration (194). Aniline, for example, fluoresces intensely in neutral form at pH 7 but is non-fluorescent in its protonated form at acid pH (196).

Sensitivity in response by two or three orders of magnitude greater than conventional ultraviolet spectroscopy can be expected from fluorescing compounds or fluorescent derivatives (195). In addition, enhanced peak identification is possible through fluorescence excitation and emission spectra using stopped flow techniques. Sylvia *et al.* (200) reported a strong correlation (mean correlation coefficient equal to 0.92) between carbon chloroform extract (CCE) and luminescence values obtained on a fluorescence spectrophotometer at selected wavelengths. The authors were unable to define the actual compound or group of compounds responsible for the fluorescent response at the selected wavelength.

Weigele *et al.* (201) recently synthesized a novel fluorogenic reagent, fluorescamine (fluram: 4 phenylspiro-[furan-2 (3H), 1'phthalan]-3,3'dione) which reacts very rapidly at room temperature with primary amines to yield highly fluorescent pyrrolinone derivatives which can be measured at picomole levels. This fluorometric method was further developed by Udenfriend (202) and has since then been used by many other researchers (170,175,192,203-205). Fluorescamine reaction with primary amines proceeds at room temperature with a half time at pH 9 of about 200-500 milliseconds for most amino acids (170). Excess reagent is hydrolyzed within seconds to water soluble nonfluorescing furanones (195). Fluorescamine, as well as its hydrolysis products, is not fluorescent. It reacts directly with amines to form stable fluorophors (390 excitation, 475 emission) whose intensity is linear with the concentration of reactant amine (170,202). Kroll *et al.* (206) found enhanced detection of naturally fluorescent compounds and fluorescent derivatives at excitation

TABLE C-10. LUMINESCENCE DATA FOR A NUMBER OF NITROGENOUS ORGANIC COMPOUNDS

Compound	Solvent	Fluorescence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
adenine	glycol-water	F		294	$\Phi = 0.06$	(197)
	water-MeOH	P	278	406	DL = 0.02	(196)
	powdered	L	260-280 296-313	355 355		(199)
adenosine	glycol-water	F		315	$\Phi = 0.003$	(197)
	EtOH	P	280	422	LD = 0.001	(196)
	powdered	L	260-280 296-313	375 375		(199)
alanine	original state	L	320-395	white		(198)
	original state	L	253	phosphoresces		(198)
aniline	water at pH 7	F	280	344	fair	(196)
	water at pH 7	F	291	361	fair	(196)
aspartic acid	original state	L	320-395	slate		(198)
ℓ-aspartic acid	original state	L	365	medium purple-blue		(198)
dℓ-aspartic acid	original state	L	365	purplish white		(198)
barbituric acid	original state	L	320-395	whitish-violet		(198)
	NaOH	L	320-295	light green		(198)
	NH_4OH	L	320-395	violet		(198)
	H_2SO_4	L	320-395	violet		(198)

(continued)

TABLE C-10. (continued)

Compound	Solvent	Fluoresence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
caffeine	EtOH	P	285	440	DL = 0.2	(196)
	original state	L	320-395	bluish white		(198)
	alkali	L	320-395	green		(198)
6-chloropurine	water-methanol	P	273	419	DL = 0.002	(196)
cytosine	glycol-water (1:1)	F		312	$\Phi = 0.06$	(197)
	powdered	L	260-280	400		(199)
		L	296-313	400		
	original state	L	365	blue-violet		(198)
guanine	glycol-water	F		320	$\Phi = 0.06$	(198)
	powdered	L	260-280	370		(199)
			296-313	370		
	original state	L	320-395	purple		(198)
	NaOH	L	320-395	light green		(198)
	NH_4OH	L	320-395	violet		(198)
	sulphuric acid	L	320-395	violet		(198)
indole	water pH 7	F	269 or 315	350		(196)
	dimethylsulfoxide	F		335	$\Phi = 0.42$	(196)
	original state	P	404			(197)
	original state	L	365	violet-blue		(198)
	sulphuric acid	L	365	green		(198)

(continued)

TABLE C-10. (continued)

Compound	Solvent	Fluorescence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
nucleic acid	NaOH	L	365	yellow green		(198)
	NH4OH	L	365	yellow green		(198)
	HCl	L	365	yellow green		(198)
nucleotide	water	L	320-395	283-380		(198)
phenylalanine	EPA	F		285		(197)
	0.5% glucose	F		290		(197)
	in water, original state	F	250-300	282	small quantum yield	(196)
	original state	F		280		(197)
	original state	F	258,205			(199)
purines	original state	F	250-275			(196)
	EPA	P		357		(197)
	water-methanol	L	272	405	DL = 0.01	(196)
pyrimidine	original state	L	320-395	yellowish		(198)
	original state	L	320-395	330-480		(198)
	water	L	320-395	310-480		(198)
	original state	F	298	326	low quantum yield	(197)
pyrrole	original state	F	240,207			(197)
	original state	L	320-395	400-480		(198)
	EtOH	L	320-395	310-480		(198)

(continued)

TABLE C-10. (continued)

Compound	Solvent	Fluorescence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
pyridine	EtOH	P	310	440	DL = .0001	(196)
2-thiouracil	EtOH	P	296	510	DL = 0.02	(196)
thymine	powdered	L	260-280	310		(199)
	powdered	L	296-313	395		(199)
	glycol-water (1:1)	F		316		(197)
tryptophan	original state	F	350	450		(197)
	original state	P	350	500		(197)
	original state	F	280	350		(197)
	original state	P	280	500		(197)
	EPA	F		325		(197)
	original state	F	280	315		(194)
	original state	F	250-300	348		(196)
	original state	F		350	$\Phi = 0.20$	(197)
	water pH 2.0	F	280,218			(199)
	water	F		348		(199)
	ethanol	P	295	440	DL = 0.002	(196)
	0.5% glucose in water,	P		435		(197)
	0.5% glucose in water	F		330		(197)
	EPA	P		490		(197)
	original state	L	320-395	grey		(198)
	phosphoric acid	L	320-395	greenish		(198)

(continued)

TABLE C-10. (continued)

Compound	Solvent	Fluorescence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
tyrosine	original state	F	280	308		(194)
	original state	F	250-300	303		(196)
	water pH 2	F	275,222			(199)
	water pH 12	F	295,240			(199)
	aqueous	F	270			(199)
	original state	F		300	Φ = 0.21	(197)
	EPA	F		305		(197)
	0.5% glucose in water	F		300		(197)
	EPA	P	395			(197)
	0.5% glucose in water	P	395			(197)
	EtOH	P	253	394	DL = 0.02	(196)
	EtOH	P	291	390	DL = 0.02	(196)
	EtOH	P	290	389	DL = 0.02	(196)
	original state	L	320-395	purple		(198)
ℓ-tyrosine	original state	L	365	strong rose-white		(198)
uracil	powdered	L	260-280	355		(199)
	powdered	L	296-313	430		(199)
	original state	L	365	violet		(198)
	NaOH	L	365	light green		(198)
	NH_4OH	L	365	blue-violet		(198)
	H_2SO_4	L	365	blue-violet		(198)
	glycol-water	L		315	Φ = 0.008	(197)
urea	original state	L	320-395	grey		(198)

(continued)

TABLE C-10. (continued)

Compound	Solvent	Fluorescence (F) Phosphorescence (P) Luminescence (L)	λ_{ex}	λ_{em}	Comments	Reference
uric acid	original state	L	320-295	medium violet		(197)

Solvent abbreviations:

MeOH - methanol
original state - compound in its original and unaltered state
EtOH - ethanol; NaOH - sodium hydroxide; HCl - hydrochloric acid
EPA - mixture of ethanol, isopentane, and ether usually in the proportion of 2:5:5
NH_4OH - ammonium hydroxide

λ_{ex} - excitation wavelength; DeMent (199) reported U.V. values only as "U.V." but indicated that this most likely represented values of 320-395 nm.

λ_{em} - emission wavelength; DeMent (199) reported color of emitted light. Approximate wavelengths are:
ultraviolet = 400 nm
violet = 400-450 nm
blue = 450-500 nm
green = 500-570 nm
yellow = 570-590 nm
orange = 590-620 nm
red = 520-760 nm
infra red =>760 nm

Comments: DL = limit of detection in µg/mL
Φ = quantum yield (or efficiency) where:

$$\Phi = \frac{\text{number of fluorescence quanta emitted}}{\text{number of quanta absorbed to a singlet excited state}}$$

$$(0 \leq \Phi \leq 1)$$

wavelengths below 250 nm but did not investigate fluorescamine derivatives. Fluorescamine has the added advantage of not being reactive with ammonia or urea (201,204), although Georidis and Coffey (90) reported a small amount of fluorescence resulting from ammonia contamination in the buffers used in their fluorometric system. Because of the extreme sensitivity to fluorescent contaminants all sampling equipment and analytical glassware must be washed in dilute hydrochloric acid and carefully rinsed prior to use (90,204). Primary amine compounds capable of forming potentially fluorescing derivatives with fluorescamine include: alanine, aspartic acid, adenine, m-aminophenol, creatine, cytosine, glycine, glycylglycine, histidine, leucine, serine, taurine, tryptophan, and tyrosine.

The utility of fluorescamine was more recently expanded by Weigele *et al.* (207), who reported that proline and other secondary amino acids could be transformed into fluorescamine responsive primary amines via oxidative decarboxylation with N-chlorosuccinimide. Felix and Terkelson (205) incorporated N-chlorosuccinimide and fluorescamine reagents into a fluorometric analyzer capable of detecting both primary and secondary amine compounds. The presence of 0.05 M hydrochloric acid in the N-chlorosuccinimide solution was required to achieve pH 2 for the oxidation of the secondary amines.

Derivatization may be accomplished either before or after the chromatographic column. In post-column derivatization the physical properties of the underivatized solutes determine their characteristic retention. In pre-column derivatization the retention values result from the properties of the derivatized solute. Although it is often assumed that pre-column derivatization tends to make a chromatographic separation more difficult, Johnson *et al.* (175) suggested that in many instances it may lead to enhanced selectivity.

KJELDAHL AND AMMONIA DETERMINATIONS

The indophenol-hypochlorite reaction for the measurement of ammonia utilizes the sensitive method of Berthelot (208) and Solorzano (209) in which ammonia is converted to a deep-blue colored compound, indophenol, by reaction with hypochlorite and phenol in alkaline solution (191). The intensity of the blue colored indophenol, measured at 635 nm, is proportional to the ammonia concentration (210). The phenol-hypochlorite reaction is extremely sensitive, so that under optical conditions µg/l levels of ammonia-nitrogen should be determinable (191,211). The method has been modified (212) by introduction of nitroprusside as catalyst. This change accentuates the blue color at room temperature.

Total Kjeldahl nitrogen includes ammonia and organic nitrogen but does not include nitrite and nitrate-nitrogen without special modification. It is measured by determination of ammonia after decomposition of the organic compounds to ammonia and CO_2 by either ultraviolet light or H_2SO_4 digestion. Ultraviolet decomposition, although potentially sensitive, gives variable recovery of organic nitrogen and, therefore, has not been widely used (191). Digestion mixtures containing a variety of catalysts and concentrations of potassium sulfate have been reported in the literature (210,211,191,213). These are shown in Table C-11. Addition of potassium sulfate to the digestion

TABLE C-11. DIGESTION MIXTURES REPORTED IN THE LITERATURE

Composition	Digestion time	Neutralized prior to ammonia determination	Reference
0.1 g SeO_2; 500 ml H_2SO_4 diluted to one liter with distilled water	approximately 1.5 hours	bromothymol blue end point (pH 6.0-7.6)	Strickland and Parsons (213)
H_2SO_4; K_2SO_4; mercuric sulfate	approximately 1 hours	phenolphthalein end point (pH 8.3 - 10.0)	Mann (211)
0.2 g SeO_2; 20 g K_2SO_4 110 ml H_2SO_4 diluted to one liter with distilled water	approximately 3.5 hours	phenolphthalein end point	Mague and Mague (191)
134 g K_2SO_4; 200 ml H_2SO_4 5 ml selenyl chloride diluted to 1 liter with distilled water	approximately 1 hour	neutralized to pH 8 or 'higher'	Scheiner (214)

mixture raises the boiling point of the H_2SO_4. The presence of phenolphthalein in the reaction mixture does not affect the final color (191). Strickland and Parsons (213) caution that ultra-pure concentrated acid must be used to ensure low blank values.

There is considerable discussion in the literature over the effect of reagent concentration, catalyst composition, temperature, reagent sequence, pH and development period on the intensity and stability of the colored indophenol (191,211,215). Copper, selenium and manganese catalysts have been employed (211,216,217). Reliable and reproducible methods for determining ammonia and Kjeldahl nitrogen in natural waters using the indophenol hypochlorite reaction, however, have been reported (213,214). Excellent reproducibility was observed by Scheiner (214) who found the maximum relative difference between duplicate determinations to be ±1%. Bolleter (218) reported standard deviations of 0.03 ppm at a level of 1 ppm of ammonia.

Although Wearne (210) reported that amino acids have an inhibitory effect on the determination, Strickland and Parsons (213) found that urea and several amino acids in the concentration of 3 μg at N/L in filtered sea water caused negligible interference. Cocking (219) found that creatine and creatinine at concentrations of 5 mg/L each did not interfere with the indophenol-blue color formation. Bolleter *et al.* (218) found that aliphatic amines, NaCl, KNO_3, $NaSO_4$, and $BaCl_2$ did not interfere. Increased absorbance resulted from the presence of copper, zinc and iron salts. Interference also resulted from the presence of aromatic amines. Manabe (220) found that at concentrations up to 5 mg/L none of 24 electrolytes tested gave rise to any significant interference in the ammonia determination. Of the 20 nitrogenous compounds tested at 0.2 mg/L amino acid nitrogen, only ℓ-cystine, ℓ-glutamic acid, ℓ-histidine, ℓ-methionine, and ℓ-phenylalanine showed interference, in the range of 5-13%. All of these compounds, however, were at concentrations substantially higher than those normally found in water. Similar results were reported by other investigators studying interference effects of electrolytes, amino acids and urea both in sea water and fresh water (209, 221-223).

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